STRUCTURE-ACTIVITY STUDIES OF POLYAMINE ANALOGUES AS ANTINEOPLASTICS

By

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(2OH)DEHSPM(R,R)	N^1,N^{14}-Diethyl-(3*R*),(12*R*)-dihydroxylhomospermine
Acetyl CoA	Acetyl coenzyme A
AdoMet	S-Adenosyl-L-methionine
AdoMetDC	S-Adenosyl-L-methionine decarboxylase
AIDS	Acquired immunodeficiency syndrome
AO	Amine oxidase
BOC	*tert*-Butoxycarbonyl
BOC-ON	2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile
BSAO	Bovine serum amine oxidase
CoMFA	Comparative molecular field analysis
DAO	Diamine oxidase
DE	Diethyl
DE(3,4,4)	N^1,N^{13}-Diethyl(3,4,4)tetraamine
DE(4,3,4)	N^1,N^{13}-Diethyl(4,3,4)tetraamine
DE(4,5)	N^1,N^{10}-Diethyl(4,5)triamine
DE(4,5,4)	N^1,N^{15}-Diethyl(4,5,4)tetraamine
DE(5,4,5)	N^1,N^{16}-Diethyl(5,4,5)tetraamine
DE(5,5)	N^1,N^{11}-Diethyl(5,5)triamine
DECroNSPM	N^1,N^{11}-Diethylcrotylnorspermine
DECroSPM	N^1,N^{12}-Diethylcrotylspermine
DEHSPD	N^1,N^9-Diethylhomospermidine
DEHSPM	N^1,N^{14}-Diethylhomospermine
DENSPD	N^1,N^7-Diethylnorspermidine
DENSPM	N^1,N^{11}-Diethylnorspermine
DEPUT	*N*,*N*'-diethylputrescine
DESPD	N^1,N^8-Diethylspermidine
DESPM	N^1,N^{12}-Diethylspermine
dcAdoMet	Decarboxylated S-adenosyl-L-methionine
DFMO	α-Difluromethylornithine
DIPHSPM	N^1,N^{14}-Diisopropylhomospermine
DIPNSPM	N^1,N^{11}-Diisopropylnorspermine
DM	Dimethyl
DM(4,5)	N^1,N^{10}-Dimethyl(4,5)triamine
DM(5,5)	N^1,N^{11}-Dimethyl(5,5)triamine
DMNSPD	N^1,N^7-Dimethylnorspermidine
DMNSPM	N^1,N^{11}-Dimethylnorspermine
DMSPD	N^1,N^8-Dimethylspermidine
DMSPM	N^1,N^{12}-Dimethylspermine
DP	Dipropyl
DP(4,5)	N^1,N^{10}-Dipropyl(4,5)triamine

DP(5,5)	N^1,N^{11}-Dipropyl(5,5)triamine
DPNSPD	N^1,N^7-Dipropylnorspermidine
DPNSPM	N^1,N^{11}-Dipropylnorspermine
DPSPD	N^1,N^8-Dipropylspermidine
DPSPM	N^1,N^{12}-Dipropylspermine
DTBHSPM	N^1,N^{14}-Di(*tert*-butyl)homospermine
eIF-5A	Eukaryotic translation initiation factor 5A
ETBHSPM	N^1-(*tert*-Butyl)-N^{14}-ethylhomospermine
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDES	N^1,N^{12}-Bis(2,2,2-trifluoroethyl)spermine
GSH	Glutathione in reduced form
GI tract	Gastrointestinal tract
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HSPD	Homospermidine
HSPM	Homospermine
IC_{50}	Concentration of drug required to reach 50% of inhibition
KB cells	A line of human epidermoid carcinoma cells
ME	Monoethyl
MEHSPM	N^1-Monoethylhomospermine
MENSPD	N^1-Monoethylnorspermidine
MENSPM	N^1-Monoethylnorspermine
MESPD(N^1)	N^1-Monoethylspermidine
MESPD(N^8)	N^8-Monoethylspermidine
MESPM	N^1-Monoethylspermine
MGBG	Methylglyoxal bis(guanylhydrazone)
MOP	3-(*N*-Morpholino)-propanesulfonic acid
MR	Multiple regression
MPNSPD	N^1-Monopropylnorspermidine
MPSPD(N^1)	N^1-Monopropylspermidine
MPSPD(N^8)	N^8-Monopropylspermidine
MPHSPD	N^1-Monopropylhomospermidine
NADH	Nicotinamide adenine dinucleotide in reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate in reduced form
NMDA	*N*-Methyl-D-aspartate
cSAT	cytosolic Spermidine/Spermine acetyltransferase
nSAT	nuclear Spermidine/Spermine acetyltransferase
NSPD	Norspermidine
NSPM	Norspermine
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
PhOH	Phenol
PIP(3,3,3)	*N*,*N*'-Bis(4-piperidinyl)-1,3-diaminopropane
PIP(3,4,3)	*N*,*N*'-Bis(4-piperidinyl)-1,4-diaminobutane
PIP(4,4,4)	*N*,*N*'-Bis(4-piperidinylmethyl)-1,4-diaminobutane
PIP(5,4,5)	*N*,*N*'-Bis[2-(4-piperidinylethyl)]-1,4-diaminobutane
PLS	Partial least squares
PLP	Pyridoxal 5'-phosphate
PUT	Putrescine

PYP(3,3,3)	*N*,*N*''-(4-pyridyl)-1,3-diaminopropane
PYP(3,4,3)	*N*,*N*''-(4-pyridyl)-1,4-diaminobutane
PYP(3,4,4)	*N*-(4-pyridyl)-*N*''-(4-pyridylmethyl)-1,4-diaminobutane
PYP(4,4,4)	*N*,*N*'-Bis(4-pyridylmethyl)-1,4-diaminobutane
PYP(5,4,5)	*N*,*N*'-Bis{2-(4-pyridylethyl)}-1,4-diaminobutane
QSAR	Quantitative structure-activity relationship
SAO	Serum oxidase
SPD	Spermidine
SPM	Spermine
SSAO	Swine serum amine oxidase
SSAT	Spermidine/spermine N^1-acetyltransferase
TFA	Trifluoroacetic acid
t.i.d.	three injections per day

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STRUCTURE-ACTIVITY STUDIES OF POLYAMINE ANALOGUES AS ANTINEOPLASTICS

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Triamine analogues have been selected as candidates for anticancer agents for high activity and low host toxicity. The research presented outlines the design and synthesis of a series of triamine analogues, their antiproliferative activities in neoplastic cell lines in vitro and in vivo toxicities in animal models.

Computer-assisted molecular modeling is used to quantify the relationship between structure and activity, based on a library of polyamine analogues with vast diversity. A model with high predictive ability is obtained and used to guide the design of triamine analogues. The triamines synthesized in this study differ in the length and spacing of their methylene backbone, and also the size of the terminal alkyl groups.

All of the compounds were screened against murine L1210 ascites leukemia in vitro by IC_{50} assay at 48 and 96 h. In an attempt to correlate this activity with some aspect of polyamine metabolism, each compound was tested for its ability to compete with spermidine for the polyamine uptake apparatus, its impact on the polyamine biosynthetic enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC), and its effect on the polyamine catabolizing enzyme spermidine/spermine N^1-acetyltransferase (SSAT) and on the polyamine pools.

The triamine analogues show highly antiproliferative activities, especially diethyl 4,5-triamine [DE(4,5)], which is highly active in both mice leukemia (L1210) and human melanoma (MALME-3M) cell lines. Acute and chronic toxicity studies showed that triamine analogues are distinctively less toxic than tetraamine analogues.

The metabolism of polyamine analogues in L1210 cells was investigated. Polyamine oxidase is responsible for the significant N-depropylation of a series of dipropyl triamines.

CHAPTER 1
INTRODUCTION

Polyamines-General

Polyamines are ubiquitous cell components essential for normal cell proliferation and differentiation (Jännë et al. 1991; Pegg & McCann 1982). They are also recognized in malignant transformation. Understanding of their biological function and the mechanism is important in the regulation of abnormal cell growth. During the last thirty years, a lot of attention has been focused on the naturally occurring polyamines, putrescine (1,4-diaminobutane), spermidine [N-(3-aminopropyl)-1,4-butanediamine] and spermine [N,N'-bis(3-aminopropyl)-1,4-butanediamine] (Figure 1-1). All of them are found in eukaryotes, while spermine is absent from most prokaryotes (Morgan 1987). Two other polyamines, cadaverine (1,5-pentanediamine) and 1,3-propanediamine (Figure 1-1) also occur in nature, but they are only occasionally found in animal tissues (Jännë et al. 1978).

Functionally, spermidine and spermine are the most important polyamines in higher eukaryotes (Pegg & McCann 1988). Polyamines have been shown to be essential for cell proliferation and differentiation in numerous studies (Jännë et al. 1991). In studies in which specific polyamine biosynthesis inhibitors partly deplete polyamines and in which polyamine-deficient mutant cells are used, cell proliferation and differentiation is halted.

Structurally, polyamines are simple aliphatic molecules composed of a linear backbone chain containing amine moieties, usually separated by three or four carbon methylene bridges. Putrescine, cadaverine and 1,3-propanediamine are diamines, and spermidine and spermine are triamine and tetraamine respectively. Because they are protonated at physiological pH (pKa's of primary amine = 8-11, pKa's of secondary amine = 9-10), polyamines are believed to behaved as polybases.

The concentrations of polyamines vary considerably between different tissues and cells (Tabor & Tabor 1964) . Rapid proliferating cells, such as intestinal mucosal cells, bone marrow and tumor cells, have a large demand for polyamines. Cells involved in polynucleotide and protein synthesis, such as proliferating cells or protein-secreting cells, contain spermidine and spermine in the millimolar range. Their concentrations also vary with the cell cycle. One of the first events in cell proliferation is induction of polyamine biosynthesis. This precedes both protein and nucleic acid synthesis (Williams-Ashman & Canellakis 1980). Under steady-state, intracellular polyamine pools are maintained within a relatively constant range by the collective effects of biosynthesis, degradation and transport , all of which are biologically specific for and elaborately regulated by the polyamines themselves (Porter et al. 1992).

Biological Roles of Intracellular Polyamines

Extensive studies show polyamines play very important biological roles, but their exact functions have not been understood very clearly because of the complexity. As polycharged molecules, polyamines provide endogenous cations and participate in regulation of the intracellular pH (Canellakis 1989). Polyamines play many biological roles in the course of their interaction with a variety of negatively charged molecules and cellular structures which include: DNA, RNA, ribosomes, proteins, membranes, etc. The following studies represent these major identified biological roles of polyamines.

Interaction of polyamines with DNA

Polyamines are believed to be important candidates in regulating DNA conformation, and for controlling DNA replication and transcription by the transformation of acetylation (Matthew 1993). In vitro, the interaction of polyamines with DNA was first shown by the ability of spermidine and spermine to precipitate DNA. Polyamines have been shown to stabilize DNA against thermal and alkaline denaturation, enzymatic degradation and shear-induced strain (Tabor 1962, Cavanaugh et al. 1984, Bachrach &

Eilon 1969, Hung et al. 1983). The interaction of polyamine-DNA complexes was seen by X-ray diffraction studies (Stevens 1970, Feuerstein et al. 1991). The stabilizing effects were attributed to neutralization of the negative charges on the phosphate groups and the consequent decrease in the level of stacking energy. At physiological concentration, polyamines can condense DNA and chromatin and promote B to Z DNA transitions (Gosule & Schellman 1978). It has been found that the structure of polyamines plays an important role in the binding of DNA and chromatin. The presence of the charges and a specific chain length of methylene ($-CH_2-$) group is required for optimal binding (Vertino et. al 1987, Balasundaram & Tyagi 1991). Acetylation of polyamines is an important mode of regulating polyamine-chromatin interaction, by diminishing the number of charges on polyamine molecules and as a result changing the helical twist of DNA caused by polyamine binding in nucleosomes.

Interaction of polyamines with RNA

Intracellular levels of polyamines and of ornithine decarboxylase markedly increase when RNA synthesis is stimulated. A dramatic example is the 1,000-fold stimulation of ornithine decarboxylase in kidney cells after androgens are administrated to castrated mice, accompanied with a substantial increase in RNA levels and a reduction in DNA content (Henningsson et. al 1978). Spermine can bind to specific sites on the rRNA molecule, and be involved both in stabilizing the conformation of t-RNA and in facilitating t-RNA splicing (Sakai & Cohen 1973, Tabor & Tabor 1984).

Effect of polyamines on protein biosynthesis

Many papers have been published on stimulation of *in vitro* protein biosynthesis by polyamines, and a few papers have addressed the effects of polyamines in vivo. The addition of polyamines results in a qualitative difference in the polypeptides synthesized in a cell-free system (Atkins et al. 1975). A combination of Mg^{2+} and polyamine in vitro results in higher incorporation of amino acids than does optimal Mg^{2+} concentration alone (Tabor & Tabor 1976, Abraham & Phil 1981). Polyamines can facilitate the association of

ribosome subunits, increase the fidelity of translation and facilitate chain termination (Rosano et al. 1983, Thompson & Kaim 1982, Hryniewicz & Vonder Haar 1983). The effects of polyamines on polypeptide chain initiation and elongation has been studied since the 1970s (Jorstad & Morris 1974, Igarashi et al. 1978, Sander et al. 1978), but there was not a clear explanation of the process until hypusine, a post-translational modified amino acid, was found.

Hypusine serves as a unique and essential amino acid constituent of the eukaryotic translation initiation factor eIF-5A (Park et al. 1993). It occurs at a single position per protein. A spermidine derivative, hypusine is formed as a post-translational modification of the eIF-5A precursor protein in the two-step process (Figure 1-2). The first step is the formation of deoxyhypusine, which involves the transfer of the 4-aminobutyl group from spermidine to lysine-50 in the eIF-5A precursor protein by deoxyhypusine synthase. The second step is the hydroxylation of deoxyhypusine by deoxyhypusine hydroxylase.

The enzymes involved in hypusine biosynthesis, especially deoxyhypusine synthase has attracted many interests as a target for growth inhibition. A couple of diamine and polyamine analogues have been reported to be good inhibitors of deoxyhypusine synthase. The spermidine binding site of this enzyme has also been defined through inhibitor studies by Jakus et al. (1993). The result provided a basis for potential control of protein biosynthesis and cell proliferation. By using guanyl diamine as an inhibitor of deoxyhypusine synthase, Park et al. postulated that the growth inhibition of Chinese hamster ovary cells was not mediated through an interference with polyamine metabolism, but through inhibition of hypusine (1994). A recent study on the cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibitor (AbeAdo) demonstrated the growth inhibition was due to the depletion of hypusine-containing form of eIF-5A, which is secondary to the depletion of spermidine by inhibition of S-adenosyl-L-methionine decarboxylase (Byers et al. 1994). All of these observations suggest that hypusine is one of the links between polyamines and growth regulation.

Functions of Extracellular Polyamines

Besides the important biological functions of intracellular polyamines, endogenous polyamines are found to have multiple effects in the central nervous system and have been suggested to be neurotransmitters or neuromodulators. One of the effects is to regulate the activity of the N-methyl-D-aspartate (NMDA) receptor channel, a subtype of glutamate receptor channels (Williams et al. 1991, Scatton 1993).

Polyamines and NMDA receptor

NMDA receptors are modulated by several endogenous ligands, including glycine, hydrogen ions, and the divalent cations Mg^{2+} and Zn^{2+}. Ransom and Stec first observed that the polyamines, spermine and spermidine, caused an increase in the binding to NMDA receptors of open channel blocker MK-801, suggesting that polyamines enhance NMDA receptor activity (1988). Because spermine does not activate NMDA receptors in the absence of glutamate and glycine, it may act on an allosteric site independent from the binding sites for these co-agonists (Ransom & Stec 1988, McGure et al. 1990).

Another action of polyamines at NMDA receptors is found as inhibitory effect. Spermine acts as either an agonist or antagonist depending on its concentration. Recent studies implied that potentiation and blocks are separate processes (Rock & Macdonald 1992, Benveniste & Mayer 1993).

The complexity of the effects of polyamines on NMDA receptors suggests that there may be more than one polyamine-binding site on the receptor channel, and at least one specific polyamine binding site has been demonstrated on the receptor complex (Benveniste & Mayer 1993, Williams et al. 1989). Eletrophysiological studies have shown that polyamines enhance NMDA receptor currents by increasing channel opening frequency and by increasing the affinity of the receptor for glycine (Rock & Macdonald 1995). On the other hand, polyamines reduce NMDA receptor currents by producing voltage-dependent reduction of single-channel amplitudes and/or by producing an open channel block. This finding represents that polyamine specific NMDA receptors could be novel therapeutic

target for the treatment of ischemia-induced neurotoxicity, epilepsy, and neurodegenerative diseases, which are believed at least partly to be caused by overstimulation of NMDA receptors.

Polyamine and gastrointestinal tract

Polyamines have relatively high concentrations in the gastrointestinal (GI) tract (Tabor & Tabor 1964). Polyamines absorbed from GI tract are a major source of exogenous polyamine, which is either acquired from diet or synthesized in the gut lumen by a high population of microbial flora (Saydjari et al. 1989). It has been found that the GI tract is a major source of polyamine for tumor growth, especially when the cellular polyamine's biosynthesis is terminated by enzyme inhibitors (Sarhan et. al 1989, Hessels et al. 1989).

Polyamines not only are essential for the regulation of intestinal growth as non-peptide growth promoting compounds (Johnson 1988), they also play a very important role in the GI tract--an inhibition effect on GI tract motility. In 1967, De Meis first reported his findings on natural polyamine's relaxing effect on intact and glycerol-treated muscle. In his study, spermidine and spermine induced relaxation of smooth muscle (guinea pig ileum and tanenia coli) which were made to contract by acetylcholine, histamine, nicotine, caffeine and excess of potassium. The inhibitory effect of synthetic polyamine analogues on gastric emptying was first found in studies of the commercial polyethyleneimines, widely used as fungicides, bactericides, antiviral and antitumor agents (Melamed et al. 1977). Further studies showed the effect of other low molecular-weight polyamine analogues on gastric emptying. Interestingly, although both spermidine and spermine very actively inhibited gastric emptying, their analogues, norspermidine and norspermine, were inactive. Triamine N-aminoethyl-1,4-diaminobutane and tetraamine N, N'-bis(3-aminopropyl) piperazine showed very low activity in inhibition of gastric emptying (Balair et al. 1981). It was clear that polyamines had a profound influence on gastric emptying and that

"endogenous spermidine and spermine may have some unrecognized GI secretomotor activity" (page 347, Balair et al. 1981).

Polyamine pharmacophore has been further investigated in Bergeron's group as an excellent candidate for construction of antitransit, antidiarrheal drugs. From a structure-activity perspective, it is obvious that very small changes in the polyamine's structure could completely eradicate the molecule's ability to inhibit gastric emptying. Among the synthetic analogs of polyamines, diethylhomospermine (DEHSPM) profoundly inhibits gastrointestinal motility in rats and this inhibition was reversed with the co-administration of bethanecol, a cholinergic agonist, but not with other pharmacologic antagonist or agonist (Sninsky et al. 1993). This drug is now studied in clinical trial phase II as a new class of antidiarrheal agents, especially in treating AIDS-related infectious diarrhea. The latest study showed that by introducing two hydroxyl groups into the aminobutyl segments of DEHSPM, a novel class of antidiarreal drug was discovered with high activity and much lower toxicity than DEHSPD (Bergeron et al. 1996).

The mechanism involved in the inhibition of GI tract motility is not quite clear at this point. Studies on other smooth muscles suggested that polyamines inhibit smooth muscle contraction at the plasma membrane which decreases the influx of calcium (Martin & Tansy 1986, Chideckel et al. 1985). In study of isolated segments of the rat small intestine, the inhibition of spermidine was sensitive to increased calcium (Chideckel et al. 1985). This phenomenon was also observed in permeabilized smooth muscle of guinea pig ileum (Sward et al. 1994).

Localization of Polyamines

Along with the investigation of polyamine biological roles, considerable effort has been exerted in order to obtain detailed knowledge of exact cellular and subcellular locations of endogenous polyamines. Regular chemical analysis of tissue sample and subcellular fractions was limited due to the diversity of cell types in most tissues and the

tendency of polyamines to redistribute during preparative procedures (Hougaard 1992). Cytochemical methods showed advantages over the chemical analysis and clearly demonstrated that in all proliferating cells and in certain differential cell types, polyamines are mostly in the cytoplasmic compartments (Hougaard 1992). In a large number of protein- or peptide-secreting cell systems, including exocrine, endocrine and neuroendocrine cell types, the polyamines are mainly localized in secretory granules and often co-localized with peptide growth factors in secretory granules. In addition, polyamines are closely associated with DNA in condensed chromatin, demonstrated by the necessity of DNase digestion prior to cytochemical staining. All of these cytochemical locations of polyamines agree with the documented biological functions mentioned above and indicate that the molecules may have many different functions during the cell cycle and in nonproliferating differentiated cells.

Polyamine Biosynthesis and Catabolism

That the levels of polyamines are elaborately regulated in the cell has been sufficiently documented. In mammalian cells, polyamines are derived from amino acids arginine and methionine (Tabor & Tabor 1984, Figure 1-3). Putrescine serves as an immediate precursor and also as a degradation and excretion product of the polyamines. In certain microorganisms and plants, the arginine is decarboxylated to form agmatine which is subsequently converted to putrescine (Luk & Casero 1987). Eukaryotes lack the enzyme which catalyzes the decarboxylation, and thus the only source of putrescine is by decarboxylating ornithine with ornithine decarboxylase (ODC) (Hayashi 1989). Ornithine is derived from the plasma or intracellular arginine by the action of arginase (Williams-Ashman & Canellakis 1979, Pegg 1986). In the cells, ornithine can accumulate to near millimolar quantities (Porter 1988).

The biosynthetic and catabolic pathways for putrescine, spermidine and spermine are well established (Tabor & Tabor 1984, Seiler 1990). There are two major pathways

along which polyamines are metabolized: the introconversion pathway and the so-called "terminal polyamine" catabolism, in which the products can not be re-utilized and are urinary excretory.

The interconversion pathway is a cyclic process which controls polyamine turnover (Figure 1-3). Putrescine is converted to spermidine and spermine through two consecutive actions of two aminopropyl transferases, spermidine synthase and spermine synthase. Both of the enzymes use decarboxylated S-adenosyl-L-methionine (dcAdoMet) as an aminopropyl donor under the action of S-adenosyl-L-methionine decarboxylase (AdoMetDC). The dcAdoMet is formed from S-adenosyl-L-methionine. Unlike putrescine, dcAdoMet is found in detectable quantities only in unperturbed cells (Porter & Bergeron 1988). The degradation of spermidine and spermine occurs first by conversion to monoacetyl derivatives, involving spermidine/spermine N^1-acetyltransferase (SSAT) and using acetyl CoA as the acetyl group donor. The acetyl derivatives are substrates for a flavin dependent polyamine oxidase (PAO), which splits the N^1-acetylpolyamines into an aldehyde (3-acetamidopropanal) and a polyamine, containing one less propylamino group. As a result, spermidine is formed from spermine and putrescine from spermidine.

Terminal polyamine catabolism is catalyzed by several kinds of amine oxidases which act at the primary amino groups. These amine oxidases include monoamine oxidase, diamine oxidase and plasma amine oxidase. By oxidative deamination of a primary amino group, each intermediate of the interconversion cycle can be transformed into an aldehyde, which is further oxidized to an amino acid or a γ-lactam (Figure 1-4). The products of the terminal catabolism are finally excreted in urinary form.

All of the amine oxidases involved can be divided into two categories, copper-containing and flavin adenine dinucleotide (FAD)-dependent amine oxidases (as shown in Table 1-1) (Morgan 1989). (1) Copper-containing amine oxidases (Cu^{2+}-containing AOs) deaminate oxidatively the primary amino group(s) of histamine, monoamine and diamine to the corresponding aldehydes (as shown blow). To this type of enzyme belong serum

amine oxidase and diamine oxidase. (2) The FAD-dependent amine oxidase is defined as a flavinprotein acting on primary amines, and usually on secondary and tertiary amines with small substituents (as shown blow). Monoamine oxidase and polyamine oxidase are FAD-dependent oxidases. In both cases the enzyme is considered to act on a CH_2-NH_2 group of the donor and oxygen is the electron acceptor.

Cleavage at the primary amino group

(operated by both Cu- and FAD-dependent AOs)

$$R\text{-}CH_2\text{-}NH_2 + H_2O + O_2 \rightarrow R\text{-}CHO + NH_3 + H_2O_2$$

Cleavage at the secondary amino group

(operated by the FAD-dependent AOs)

$$R\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}R' + H_2O + O_2 \rightarrow R\text{-}CHO + H_2N\text{-}CH_2\text{-}R' + H_2O_2$$

or $$R\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}R' + H_2O + O_2 \rightarrow R\text{-}CH_2\text{-}NH_2 + HOC\text{-}R' + H_2O_2$$

Enzymes in Interconversion Pathway

Three rate-limiting enzymes, ornithine decarboxylase (ODC, EC 4.1.1.17), S-adenosyl-L-methionine decarboxylase (AdoMetDC, EC 4.1.1.50), and spermidine/spermine N^1-acetyl-transferase (SSAT), are the key enzymes that regulate polyamine metabolism.

First, rate-limiting enzymes are recognized by their low activity. Studies show that the three enzymes listed above have the lowest activity among all of the enzymes involved in polyamine metabolism (Seiler 1990). The activity of these regulatory enzymes can change rapidly under different circumstances by different mechanisms. Second, these three enzymes are regulatory enzymes which turn over quickly, with biological half-lives between 20 and 40 min (Russell & Snyder 1969, Seyfried et al. 1982, Matsui & Pegg 1981).

The ODC has been isolated from a considerable array of species. In all cases, it has been found to be dependent on pyridoxal 5'-phosphate (PLP) for activity. Studies of ODC from different mammalian sources showed over 90% identity in amino acid sequences (McCann & Pegg 1992). In general, induction of ODC is either physiological (hormones, growth factors) or non-physiological (tumor promoters, toxic agents). The induction of ODC is mainly from enhanced transcription and protein synthesis (Porter and Bergeron 1988). However, the decreased enzyme activity is associated with post-transcriptional regulation. The identified mechanisms involve either reduced translation of ODC mRNA or increased activity of an ODC-directed protease.

The second regulatory enzyme, AdoMetDC, is an unusual decarboxylase in the way that it uses the covalently bound pyruvate as a prosthetic group instead of using pyridoxal phosphate (Pegg 1984). Then, the carbonyl group of pyruvate forms a Schiff base with the substrate (Van Poelje & Snell 1990). Accelerated activities of AdoMetDC is considered to be mediated by increases in enzyme synthesis, in half-life and in transitional efficiency of mRNA. Alternatively, inhibition of mRNA translation is believed to be responsible for the reduction of AdoMetDC related to higher levels of intracellular polyamines (Porter & Bergeron 1988).

The third regulatory enzyme, SSAT, was recognized simply as a acetyltransferase when monoacetyl derivatives of polyamines were first found in brain cells and urine 30 years ago (Libby 1980). The association of SSAT with the metabolic interconversion of polyamines was not realized until N^1-acetylspermine was found to be an extremely efficient substrate for rat liver cytoplasmic polyamine oxidase (PAO) (Bolkenius & Seiler 1981). The latter oxidatively eliminated acetylated aminopropyl units of SPD or SPM by their conversion to 3-acetamido-propanal. In this way, PAO converted the N^1-acetylspermine to spermidine and N^1-acetylspermidine to putrescine (Hölttä 1977). There are three forms SSAT capable of the acetylation of polyamine: nuclear acetyltransferase (n-SAT), noninducible cytosolic acetyltransferase and inducible cytosolic acetyltransferase (c-SAT)

(Seiler 1987). The first two are not inducible, while the last one, as its name suggests, is highly inducible. The high inducibility of cytoplasmic SSAT was first reported in livers exposed to carbon tetrachloride (Matsui & Pegg 1980). Numerous in vitro and in vivo systems respond to a wide variety of physiological, pathological and pharmaceutical stimuli (Seiler 1987). Among the pharmacological stimuli, the antiproliferative agent, methylglyoxal bis(guanylhydrazone) (MGBG) (Persson & Pegg 1984, Nuttall & Wallace 1987) and some polyamine analogues are potent inducers of SSAT. i.e. diethylnorspermine (DENSPM) induced SSAT activity 15 times over unstimulated level after a 48 h treatment of murine leukemia cell at 2 μM (Bergeron et al. 1994, Libby et al. 1989).

In contrast to the regulatory decarboxylases and acetyltransferase, spermidine and spermine synthase are usually present in cells and tissues at high levels of activity. They are stable proteins with biological half lives of several days (Seiler 1990).

Polyamine oxidase (PAO) is also a stable enzyme. It contains a tightly bound FAD prosthetic group and acts on the secondary amine group of N^1-acetylpolyamine derivatives of spermidine and spermine yielding spermidine and putrescine, respectively, along with 3-acetamido-propanal and hydrogen peroxide. Although this enzyme is also able to transform spermine into spermidine, and spermidine into putrescine in the presence of benzylaldehyde, it has demonstrated that N^1-monoacetylspermidine, N^1-monoacetylspermine and N^1, N^{12}-diacetylspermine are much better substrates for PAO than the non-acetylated polyamines (Bolkenius & Seiler 1981).

Enzymes in Terminal Pathway

Three types of amine oxidases have been found involved in the terminal pathway. They are monoamine oxidase, diamine oxidase and serum oxidase. All of them act on the primary amino groups and generate aldehyde, hydroxyperoxide and ammonia. These products are believed to be related to both in vitro and in vivo toxicity of polyamines (details in Chapter 2).

The monoamine oxidase is a flavin-dependent amine oxidase found in mitochondria. It has been widely investigated for its primary involvement in the metabolism of the biogenic monoamine neurotransmitters. It only plays a role of secondary importance in the oxidation of acetylated polyamines (Mondovì et al. 1989). The polyamine substrates of MAO are listed in Table 1-1.

Diamine oxidases (DAO) are Cu^{2+}-containing enzymes localized in mitochondria and microsomes. They are particularly active towards aliphatic diamines (e.g. putrescine, cadaverine, Table 1-1) and histamine. The DAOs also catalyze the deamination of SPD, SPM and their derivatives as long as there are primary amino groups available (Table 1-1).

Serum oxidase (SAO), the copper-containing amino oxidase, which is especially rich in ruminant serum, was the first polyamine-selective oxidase to be described (Hirsch 1953). Among the SAOs, bovine serum amine oxidase (BSAO) and swine serum amine oxidase (SSAO) are the best characterized (Morgan 1989, Mondovì et al. 1989). Both enzymes catalyze the oxidative deamination of primary amino groups of several aliphatic and aromatic monoamines, but while BASO oxidizes spermidine and spermine, SSAO does not. The polyamine substrates of SAO is shown in Table 1-1.

Polyamine Transport

Polyamine transport system is sensitively regulated by polyamine levels in the cells. Along with the polyamine biosynthesis and catabolism pathways, it maintains the intracellular polyamine pools at a relatively constant level.

Polyamine transport system has been found in a wide range of mammalian cells and it is believed to be protein nature (Kramer et al. 1993). Through kinetic studies in most systems, the putative transport protein has been shown to be saturable and distinctly energy dependent with separate sodium-dependent and sodium-independent components (Seiler & Dezeure 1990).

Polyamine uptake is essential and sufficient to sustain cell growth fully in cells where the biosynthetic pathway is genetically defective or drug inhibited (Pilz et al. 1990, Kramer et al. 1989). Under such conditions, cells may up-regulate their polyamine transport system in order to ensure that intracellular pools are maintained (Alhonen-Hongisto et al. 1980). On the other hand, in order to avoid toxicity associated with polyamine excess, the uptake mechanism may also be down regulated in response to a surplus of polyamines (Kankinuma et al. 1988, Byers & Pegg 1990).

The substrate for the polyamine uptake system is not strictly limited to naturally occurring polyamines. A wide variety of compounds are known to be transported by this system, including synthetic molecules such as the herbicide, paraquat (Figure 1-5) (Byers et al. 1987), the SSAT inhibitor, methylglyoxal-bis(guanylhydrazone (MGBG) (Dave & Caballes 1973, Porter et al. 1982) and series of polyamine analogues (Bergeron et al. 1988 and 1989, Byers & Pegg 1990).

The polyamine transport system also responds to various physiological conditions. The activity of the system increase during hormonal stimulation and proliferation and decreases during differentiation (Rinehart & Chen 1984). While under conditions that disfavor proliferation, for example, achievement of confluence, absence of growth factors or existence of inhibitors, there is a substantial efflux of polyamines, particularly spermidine, from the cell (Jännë et al. 1983).

Like polyamine uptake, polyamine depletion also plays an important role in maintaining the polyamine pool level. In vitro studies, a massive efflux of spermidine was detected in a short 24 h period, when cells were exposed to polyamine analogues. Spermine, different from spermidine, can not be excreted from cells as free amine. It needs to be acetylated by the action of acetyltransferase before depletion. Acetylation reduces the polyamine's charge and so results in weaker binding in various cellular compartments. In whole animal studies, free polyamines account for about 75% of the polyamines found in

rat urine, while humans excrete the polyamines nearly exclusively as monoacetyl derivatives (Seiler et al. 1981).

Polyamines and Cancer

As polyamines are related to proliferation, differentiation and regeneration of normal cells, not surprisingly, they are also recognized in malignant transformation. As early as 1853, it was reported that leukemic spleen was rich in spermidine (Chartot & Robin). This observation was extended almost a hundred years later by Hämäläinen who found increased concentrations of spermine in the liver and bone marrow of patients that had died of leukemia (Jännë et al. 1978). In 1971, Russell et al. reported that patients with a variety of tumors had elevated levels of polyamines in their urine. Since then, polyamines and their acetylated-forms have been widely studied as markers for malignant tumors. However, the usefulness of polyamines as markers is limited to only several malignant diseases: breast, prostatic, bladder and colon cancers (Horn et al. 1982, Czuba & Smith 1991). Although polyamines can not be used in screening for malignancy because of nonspecific elevation, the polyamine assays are found valuable to monitor the long term progression or regression of tumors and to evaluate short-term efficacy of therapy.

The polyamine biosynthetic pathway has attracted a lot of interest as a therapeutic target. Enzyme inhibitors with the ability to interfere with polyamine biosynthesis were first chosen as therapeutic agents. Numerous studies of ornithine decarboxylase and the therapeutic effect of inhibition of ODC occurred in the late 1970s and 1980s. The most widely used specific inhibitor of ODC, α-difluoromethylornithine (DFMO) (Figure 1-6), leads to a major reduction of putrescine and spermidine. As a result, growth is significantly reduced (Pegg & McCann 1988b, Marton & Pegg 1995). However, in most cases, DFMO only causes a small reduction in spermine. Like other specific inhibitors, the effects of DFMO are cytostatic instead of cytotoxic. It is suggested that the lack of cytotoxicity may be due to the residual spermine in the cells. The atom of DFMO as a

irreversible inhibitor starts with its recognition as a substrate by ODC, generating a highly reactive intermediate by decarboxylation of DFMO, and finally formation of a covalent bond with the protein (Metcalf et al.1978, McCann & Pegg 1992). The actual sites of DFMO binding have been identified (Poulin et al. 1992). The major site of binding was the cysteine residue at position 360 and is contained in a highly conserved region of ODC in the peptide -GPTCD- found in all known eukaryotic ODC sequences. This site accounts for about 90% of the total binding. It has been suggested that cysteine 360 may be located close to the active site of the enzyme.

DFMO has shown significant efficacy as a single agent in slowing the growth of tumors cells in vitro and in many animal models in vivo as a single agent (Sunkara et al. 1987). However, DFMO did not seem to significantly affect tumor growth or progression of diseases in more than 500 patients with a variety of malignancies (Schecter et al. 1987)

The first specific inhibitor of AdoMet is MGBG (Figure 1-6), introduced by Williams-Ashman and Schenone (1972). It exerted significant enzyme inhibition, both in vitro and in vivo (Pegg 1988). However, its usefulness is limited because (1) it is a reversible inhibitor; (2) it is not absolutely specific and also inhibits some other enzymes, such as diamine oxidase; and (3) it is highly toxic (Pegg & McGill 1978). The discovery of MGBG led to the design and synthesis of a number of specific AdoMetDC inhibitors, which were expected to provide potent antiproliferative agents (Figure 1-6) (Pegg & McCann 1992). However, like MGBG, the inhibition effects of these agents generally lead to a large increase in putrescine and a decline in spermidine and spermine. As putrescine content rises by more than the decrease in the spermidine and spermine levels, the total supply of polyamines actually increases as the inhibitor is applied. In cell culture, the growth inhibitory effects could be completely abolished by simultaneous administration of spermidine (Kramer et al. 1989, Regenass et al. 1992). For this reason, the therapeutic values of these inhibitors may be limited due to the reversal of growth inhibition by exogenous spermidine.

There are significant problems with the use of inhibitor of polyamine biosynthetic enzyme: (1) the key regulatory enzymes in the biosynthesis--ODC and AdoMetDC are under the feedback regulation of polyamine levels. Depletion of polyamine level will induce a large compensatory increase in these enzyme activities and uptake of exogenous polyamines, which in turn will decrease the inhibition; (2) turnover rate of these enzymes is so fast, the cancer cell is able to overcome the block quickly, thus making the inhibition transient. This explains the limited clinical value of these enzyme inhibitors, at least when they are used as a single agent.

Polyamine analogues, the laboratory constructed imitation polyamines, have attracted considerable attention during recent years, as an alternative to direct enzyme inhibitors. Although polyamine analogues can not substitute natural polyamines in cell proliferation, they are accepted by the cell as the natural polyamines in many circumstances. When facing a substantial amount of pseudo-spermidine or spermine, cells take in the "ready-made nutrition" and at the same time shut down their own polyamine synthesis to save energy. Intracellular natural polyamine are then excreted or degraded to keep the balance of polyamine pool.

Compared to the enzyme inhibitors, polyamine analogues have several advantages as a regulatory approach to interfere with polyamine biosynthesis: (a) polyamine analogues use the specific cellular polyamine transport apparatus; (b) simultaneous regulation of different enzymes can be managed, including suppression of biosynthetic enzymes and induction of catabolic enzymes; (c) compensatory increases in related enzymes do not occur as with enzyme inhibitors; (d) compensatory increase in cellular uptake of polyamines do not occur; (e) depletion of all polyamine pools, including spermine, is possible; (f) structure modification of polyamine analogues can make them immune from acetylation by SSAT and have a much longer half-life than the natural polyamines. As a result, the analogues can be concentrated up to 1000 times more in the cell than in the environment.

This is one of the reasons for high activity of polyamine analogues at a very low μM concentration (Porter & Bergeron 1988).

The first series of polyamine analogues was linear aliphatic triamines and tetraamines homologues of spermidine and spermine (Israel et al. 1964). Antitumor activities were found both in vitro against human epidermoid carcinoma (KB) and in vivo against transplantable mouse tumors. Among the analogues, a spermine analogue, N,N'-bis(3-aminopropyl)nonane-1,9-diamine was found to be most active. In 1981, Weinstock et al. demonstrated that some of the homologues and acylated derivatives of spermidine and spermine were active against B16 melanoma and human epidermoid carcinoma of the nasopharynx.

It was observed that when polyamine biosynthesis was shut down in transformed cells by treatment of DFMO, the cells incorporated spermidine at an accelerated rate relative to untreated cells (Jännë et al. 1978). This encouraged some researchers to consider the spermidine uptake apparatus as a means of delivering antineoplastic drugs to transformed cells (Porter et al. 1985). It was found that the uptake of MGBG (an AdoMet inhibitor), an antineoplastic, also occurred via the polyamine transport apparatus and was enhanced by pretreatment of the cells with DFMO (Casero et al. 1984). Noticing the limited structural similarity between MGBG and spermine, Porter and Bergeron suggested that the polyamine transport system might tolerate some structural modification on natural polyamines. This reasoning led to the design and synthesis of a new series of polyamine analogs, N^4-spermidine and N^1,N^8-spermidine derivatives (Porter et al. 1982 and 1985). It has been found that (1) terminal alkylated spermidine analogues have much higher activity than the acetylated ones; (2) antiproliferative activity is higher when alkylation at terminal nitrogens (N^1 and N^8) than when it is at the middle nitrogen (N^4); (3) uptake is dependent on the availability of the primary amines. Among all of the analogues, N^1,N^8-diethylspermidine (DESPD) was found to be the most active. After treatment of 96 h at 10 μM, DESPD depleted intracellular putrescine and spermidine, reduced spermine by 50%

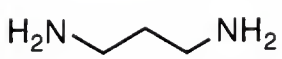
and decreased ornithine and S-adenosylmethionine decarboxylase by 98%. The regulation mechanism of DESPD on ODC was the same as that of spermidine, which decreased the enzyme activity by down regulation rather than direct bind and inactivate the enzyme (Porter et al. 1986). Studies also indicate that while sharing the same cellular uptake system, DESPD can not substitute for spermidine to support cell growth (Porter & Bergeron 1988).

The diethyl derivatives of putrescine and spermine were also tested for their ability to regulate polyamine biosynthesis and inhibit L1210 leukemia cell growth (Porter et al. 1987). The antiproliferative activities are in the order of diethyl spermine (DESPM) > DESPD > diethyl putrescine (DEPUT). The DESPM also had significant effect on polyamine depletion and enzyme inactivation. As the tetraamine analogues showed the highest activity, more diethyl alkylated tetraamines were investigated as antineoplastics (Bergeron et al. 1989). The order of antiproliferative activity in vitro was shown to be DEHSPM > DESPM > DENSPM. In vivo studies, in human pancreatic adenocarcinoma models, DENSPM showed greater antitumor activity than either DEHSPM or other conventional agents (Chang et al. 1992). The DENSPM also demonstrated remarkably high inhibition to the growth of MALME-3M and SN-I melanomas; A549 lung adenocarcinoma; and A121 ovarian carcinoma (Marton & Pegg 1995). It is believed to be a very promising anticancer drug, and its phase II trials are in process.

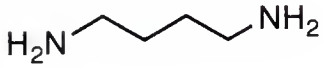
A systematic investigation is needed in order to first define the minimal structural requirements of a polyamine analogue necessary for antineoplastic behaviors. Once having done this, we can then modify nonessential components of the molecule possibly to minimize toxicity. It is clear that the structural changes in polyamine analogues are closely related to their differences in biological activities. In order to study the relationship between structure and activity, a library of polyamines, especially tetraamines, has been constructed in Dr. Bergeron's lab. Extensive studies have been conducted to find the correlation between biological activities and the following structure factors: (1) charge

distribution, (2) distance (methylene backbone) between the nitrogen atoms, (3) changes of size in terminal alkylation groups (Bergeron 1995a, 1989 and 1994).

In this study, a number of tetraamines were synthesized and tested for their antiproliferative activities, their impact on polyamine pools, the effect on polyamine regulatory enzymes (including ODC, AdoMet and SSAT) and their ability to compete with radiolabelled spermidine for polyamine uptake apparatus. All of the tetraamine analogues, including the ones synthesized in current and from previous work, were used to draw a clear picture of structure and activity relationship by computer-assisted molecular modeling. In light of the low toxicity of triamine analogues compared to tetraamine analogs, a series triamine analogues were designed, synthesized and tested for antiproliferative activity. Computer-assisted molecular modeling was used to guide the design.

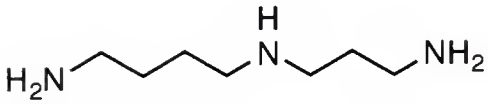


1,3-propanediamine

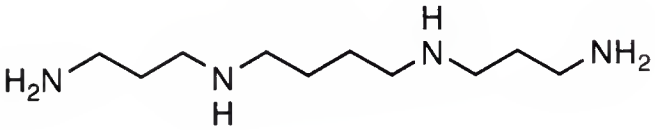


putrescine

cadaverine



spermidine



spermine

Figure 1-1: Structure of natural polyamines.

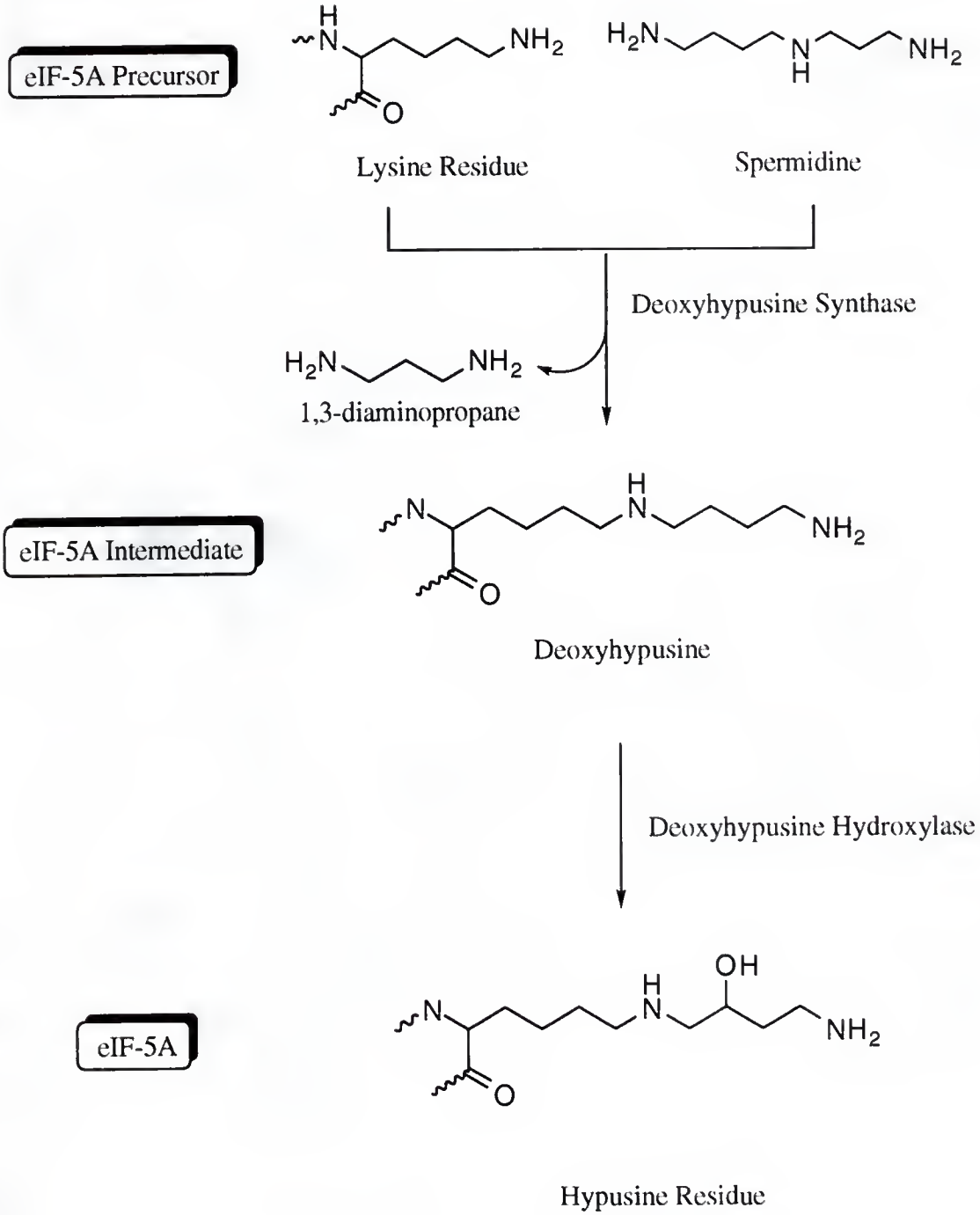


Figure 1-2: Formation of hypusine.

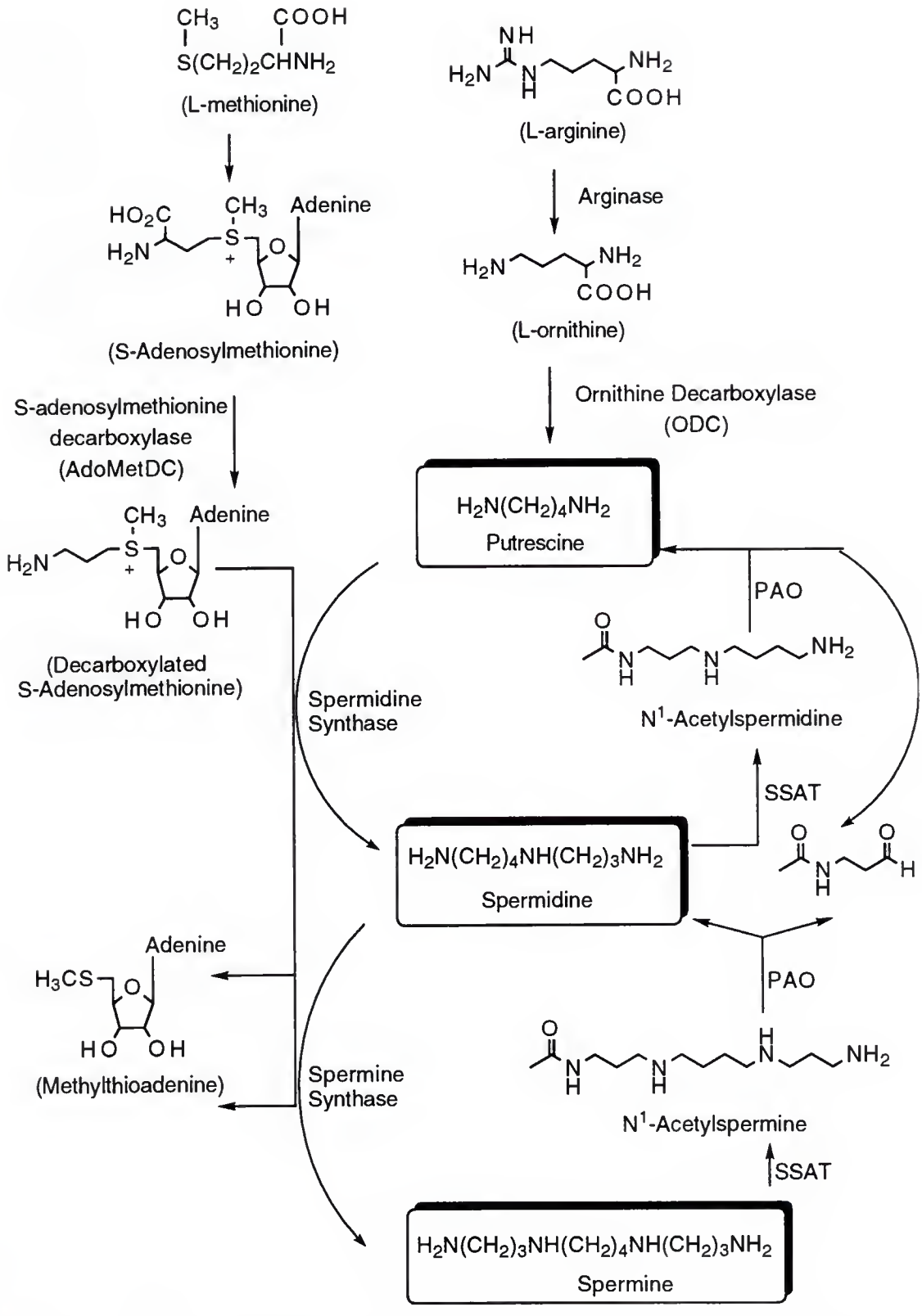
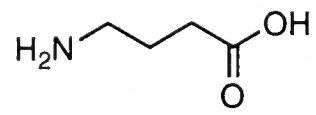
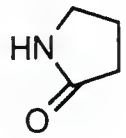


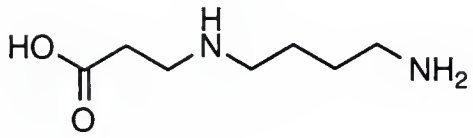
Figure 1-3: Polyamine biosynthesis and the interconversion pathway of catabolism.



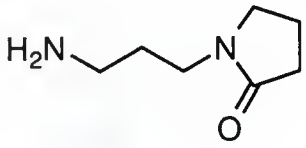
4-Aminobutyric acid



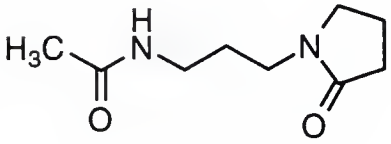
Pyrrolidin-2-one



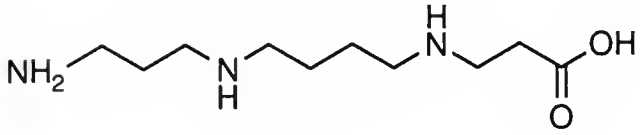
Putreanine



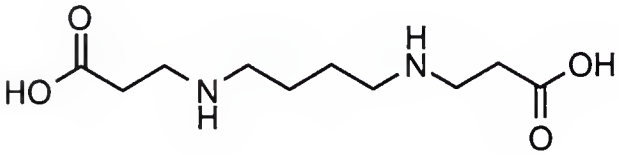
Isoputreanine lactam



N-Acetyl isoputreanine lactam



N^8-(2-Carboxyethyl) spermidine



Spermic acid

Figure 1-4: Metabolites of polyamines formed in the terminal polyamine catabolism pathway through oxidative deamination.

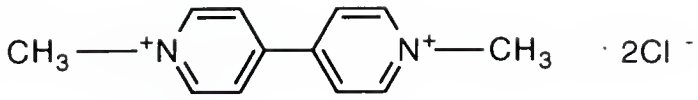
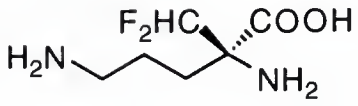


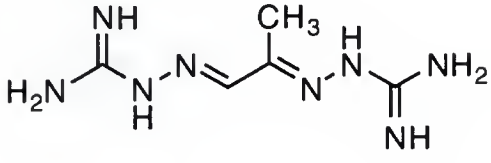
Figure 1-6: Structure of paraquat, a kind of herbicide which transported by polyamine transport apparatus.

ODC Inhibitor

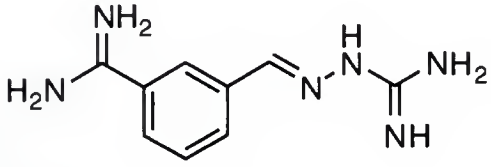


α-Difluoromethylornithine
(DMFO)

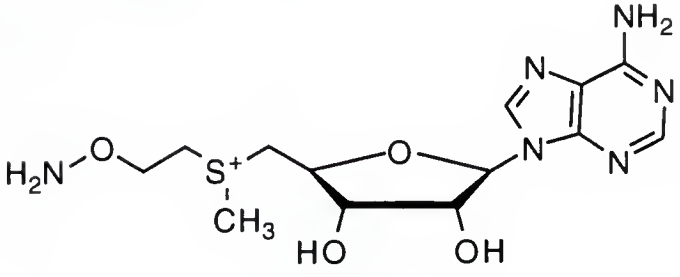
AdoMetDC Inhibitors



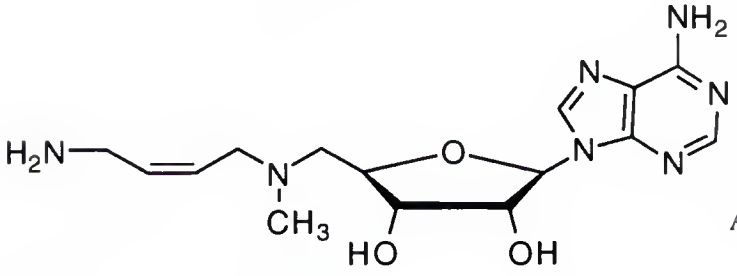
MGBG



CGP-39'937



MHZPA



AbeAdo

Figure 1-6: Structure of ODC inhibitor and AdoMet inhibitors.

Table 1-1: Different polyamine substrates of mammalian amine oxidases.

Polyamines and Acetylated Polyamines	Flavin-dependent Proteins		Copper-dependent Proteins	
	MAO	PAO	DAO	SAO
Putrescine			+	
Acetylputrescine	+			
Cadaverine			+	
Acetylcadaverine	+			
Spermidine		+		+
N^1-Acetylspermidine		+	+	
N^8-Acetylspermidine	+			+
Spermine		+		+
N^1-Acetylspermine		+		+

+ Represents the polyamine or acetylated polyamine is a substrate of the amine oxidase.

CHAPTER 2
TRIAMINES AS ANTIPROLIFERATIVE AGENTS

Design Concept

In this study, a group of triamine analogues is designed as a new series of anticancer agents. The rational is based on the following facts: (a) triamine analogues have high antiproliferative activities; (b) triamine analogues have significantly lower host toxicity than tetraamine analogues.

Antiproliferative Activity of Triamines

During the 1980s, several of spermidine analogues were investigated for their antiproliferative activity by Porter et al. (1982, 1985). The two dialkylated spermidine N^1,N^8-bis(ethyl)spermidine and N^1,N^8-bis(propyl)spermidine appeared be the most active analogues. At 48 h, a 50% inhibition of growth (IC_{50}) was reached at 40 and 50 μM. They also significantly reduced the polyamine pools and biosynthetic enzyme activities at 10 to 30 μM. Among different kinds of modification, terminal dialkylation was shown to be the most effective one in the terms of increasing antiproliferative activity.

The spermidine homologues, norspermidine (NSPD), homospermidine (HSPD) and 4,5-triamine (Figure 2-1) are found in some plants and bacteria, but not in mammalian cells (Hamana et al. 1994, Kuttan et al. 1971, Hamana & Matsuzaki 1990, Fulihara et al. 1995). In vitro these unusual triamines are able to stimulate polypeptide synthesis of *E. coli* at suboptimal Mg^{2+} concentrations in a manner comparable with that of spermidine (Koumoto et al. 1990). In eukaryotic cells, Porter and Bergeron found that several spermidine homologues (including NSPD and HSPD) could be transported into the cells and

substrate SPD for cell proliferation, except NSPD because of inherent toxicity (1983). Later on, Sunkara et al. investigated the antitumor activity of NSPD (1988). It is obvious that these natural triamines, along with the synthetic triamine, 5,5-triamine (Figure 2-1), could be good candidates for a source of the triamine backbones for modification. As in the previous study, terminal dialkylation is used here as an effective derivatization of triamines to provide potent antiproliferative agents with minimal toxicity. In order to investigate the relationship between the size of the terminal groups and activity, alkylating groups were designed to vary from methyl, ethyl to propyl.

Design of Polyamine Analogues with Low Toxicity

In order to design new polyamine analogues with lower toxicity, the historical studies of polyamine toxicity, both in vitro and in vivo were reviewed. A new series of polyamine analogues were designed and anticipated to have lower toxicity than other polyamine analogues.

Previous study of polyamine toxicity in vitro

Although polyamines are essential for growth, at high concentrations, spermine and spermidine are toxic to several species of bacteria, bacteriophage (Hirsch 1953, Kimes & Morris 1971) and a number of different mammalian cell lines (Gaugas & Dewey 1978, Ali-Osman & Maurer 1983, Parchement et al. 1990). The toxicity, often found with the presence of ruminant sera in the culture medium, is believed to depend on the activity of serum amine oxidases (SAO) which break the polyamines into highly active fragments and cause the damage to the cell. Since ruminant is a component of many tissue culture media, oxidative deamination of the polyamines by SAO was believed to be a frequent source of erroneous interpretation of experiments with cultured cell.

As mentioned in the introduction, amine oxidase is a family of enzymes that include monoamine oxidase, diamine oxidase (histaminase), polyamine oxidase and serum amine oxidase. Amine oxidases have the generation of hydrogen peroxide and aldehyde in

common. Diamine oxidase and serum amine oxidase are Cu^{2+}-dependent amine oxidases. Monoamine oxidase and polyamine oxidase are FAD-dependent amine oxidase.

The oxidation of polyamines by serum amine oxidase is represented as in Figure 2-2. These enzymes oxidize spermine and spermidine only at terminal-CH_2NH_2 groups with the formation of the corresponding aldehydes plus NH_3 and H_2O_2. The aldehydes are then transformed into lower order amines by spontaneous β-elimination of acrolein or turned into amino acids by oxidation. The aminoaldehydes, peroxide and acrolein are postulated to be responsible for the toxicity of extracellular polyamines in cell culture.

Toxicity mediated by aminoaldehyde. In 1964, Tabor et al. first reported the identification of aminoaldehyde produced by oxidation of spermine and spermidine with purified plasma amine oxidase (1964). Dioxidized spermine ($t_{1/2}$ 2.3 h in tissue culture medium) led to a potent non-cytotoxic arrest of cell proliferation confined at the G_1 phase of the cell cycle (Gaugas & Dewey 1978). The aminomonoaldehyde [N^1-(4-aminobutyl)-aminopropionaldehyde)] and the dialdehyde [N^1, N^4-bis-(3-propionaldehyde)-1,4-diaminobutane] are found to interfere with DNA replication and cross-link DNA in vitro (Bachrach 1973, Eilon & Bachrach 1969). By cross-linking a double-strand DNA, the aminoaldehydes may prevent the strand separation necessary for transcription and replication, in a manner similar to other bifunctional alkylating agents. Aminoaldehydes may also exert their toxicity by interaction with proteins. Aldehydes are known to bind reversibly to the amino group of amino acids, to the basic residues of proteins, and to combine irreversibly with the sulphydryl groups of cysteine (Schauenstein 1978).

Toxicity mediated by peroxide. Henle et al. observed the random induction of DNA strand breaks as a result of spermidine oxidation by serum amine oxidase in Chinese hamster ovary cells (1986). Damage from SPD can be reduced when culture media are supplemented thiourea (15 mM) or catalase (1000 units/mL) (the enzyme evolved in removal of H_2O_2).

Toxicity of acrolein. Acrolein was found as a product of enzymatically oxidized spermidine or spermine by Alarcon in 1970. At low levels (0.01-0.02 mM), acrolein was able to inhibit nucleic acid and protein synthesis in E. coli. Alarcon suggested that acrolein, the highly active aldehyde, was the oxidation product largely responsible for the polyamine's cytotoxicity on mammalian cell based on its high cytotoxicity and high affinity for SH groups (important in cell division and preferential inhibition of nucleic acids). Acrolein is formed from spermidine and spermine but not putrescine (Alarcon 1972). The fact that acrolein was formed from spermidine and spermine instead of putrescine could be correlated with the observation that in rat cerebellar cultures, the neurotoxicity of spermidine and spermine were much more potent than putrescine by about two orders of magnitude (Gilad & Gilad 1987).

Early studies showed that replacement of calf serum by horse serum and addition of aminoguanidine could abolish the toxicity of polyamine in cell culture (Gahl & Pitot 1978). Aminoguanidine, as a copper chelator, is a highly effective inhibitor of serum amine oxidase and diamine oxidase present in fetal calf serum. Ruminant sera, especially bovine sera, contain high titers of serum amine oxidase (Bachrach 1970). Horse sera have lower levels of amine oxidases. Rat serum is known to contain very low amine oxidase activity (Seiler et al. 1980). Human sera may have no or very low levels of amine oxidase.

Although in many cases, the toxicity was understood to be due to the extracellular metabolism of spermine by the amine oxidases found in bovine serum, some studies have shown that the toxicity is not entirely dependent on extracellular oxidation, but may be due in part of an intracellular event. Higgins et al. suggested that other factors were involved in the toxicity by studying the growth of KB cells in different types of sera (1969). It was found that the inhibition was decreased as calf and bovine sera concentration increased, while an increase in horse serum concentration led to higher inhibition. Other studies show that polyamines are also toxic in the absence of ruminant serum. Smith et al. found that in a serum-free system, the oxidase activity on the cell surface reacted with polyamines lost

from the cell, and this was attributable to the growth inhibition of T-lyphocytes and granulocytes (1983). The inhibition was reversible in the presence of polyamine oxidase inhibitor 3-hydroxybenzyloxyamine. The cellular enzyme, the FAD-dependent polyamine oxidase, is believed to be responsible for the intracellular oxidation of polyamines. It acts on spermidine and spermine to produce 3-aminopropionaldehyde, H_2O_2 and putrescine, although the preferred substrates of this enzyme are the acetylated polyamines (Morgan 1989).

Brunton et al. studied the dose-dependent inhibition of spermine in BHK-21/C13 cells grown in medium supplemented with horse serum (1989a, 1989b). At toxic levels DNA synthesis was decreased and depletion of intracellular glutathione was observed. It was postulated that the toxicity may result from the loss of intracellular GSH. But at this point, it's not quite clear whether the loss of GSH is simply due to the presence of oxidants or due to the block of GSH biosynthesis.

Polyamine analogues are less toxic than the parent polyamines in vitro. Porter et al. found that in the cell culture with presence of fetal bovine serum, the terminal dialkylated polyamines analogues did not show the potent nonspecific cytotoxicity as their parent polyamines (1987b). Thus, amino alkyl substitution was suggested to be an effective means of eliminating nonspecific host toxicity otherwise associated with enzymatic oxidation of polyamines.

Previous study of polyamine toxicity in vivo

Compared to the in vitro studies, there are fewer descriptions of the toxicity of spermidine and spermine and their mechanism on the intact animal. In 1956, the first pharmacological study of polyamines in laboratory animals was reported (Tabor & Rosenthal 1956). It was found that the acute toxicity of spermine and spermidine was primarily related to renal tubular necrosis. Spermidine is approximately one-twentieth as nephrotoxic as spermine. Less charged polyamines like putrescine and cadaverine are not nephrotoxic. The order of toxicity is tetraamine > triamine > diamine. The same trend was

also found in the acute toxicity of polyamines in mice (norspermine > norspermidine > 1,3-diaminopropane) (Bergeron et al. 1995b), which agreed with the result found in the cell culture of resting lymphocytes (toxicity of spermine > spermidine > putrescine) (Nishida & Miyamoto 1986).

The pharmacological properties of spermidine and spermine was further explore by Shaw and the dose dependent toxicity was carefully studied in mice and rat (1972). The acute toxicity cause by intravenous injection of spermine were ataxic, sedation, ptosis, piloerection, hyperthermia, cardiorespiratory failure and lethargy. The toxic effect of spermidine was similar to that of spermine but larger doses were required to produce equal effects. From the acute LD_{50} value, spermidine was at least three times less toxic than spermine.

Design Concepts

From all of the studies above, triamines consistently showed lower toxicity both in vitro and in vivo. This phenomenon can be explained by the fact that under the effect of polyamine regulatory system, excess diamines and triamines can be excreted from the cell easily, while tetraamines are only poorly exported from cells unless acetylated and oxidized to SPD under the successive actions of acetylase--SSAT and polyamines oxidase. It is possible that longer half-life of spermine make it more toxic to the host than spermidine.

Compared with the natural polyamines, the terminal alkylated analogues have lower acute toxicity. It was reported that DESPM was about 50% less toxic than spermine for mice after a subcutaneous injection (Igarashi et al. 1990). The absence of primary amine in the alkylated analogues may reduce the host toxicity associated with enzyme oxidation of polyamines (Porter et al. 1987b).

The studies of chronic toxicity of polyamine analogues focused mainly on tetraamine analogues. Among them, DEHSPM is the most toxic one with chronic ID_{50} of

37.5 mg/kg[chronic multiple-dose ip (t.i.d. x 6 days)] (Bergeron et al. 1994). Part of its toxicity is due to its ability to initiate inhibition on gastrointestinal tract mobility.

It was found that the alkylated tetraamines were preferentially accumulated over the native polyamines in L1210 cells (Bergeron et al. 1989). While significantly inhibited the growth of cancer cells, the tetraamine analogues may have some disturbance on the regular cell proliferation due to their prolonged existence in the host system.

Based on the previous studies of polyamine toxicity, triamines analogues are expected to show lower toxicity than the tetraamine analogues. The triamine analogue with the highest antiproliferative activity will be studied for its acute and chronic toxicity, along with its effect on GI tract.

QSAR Study of Polyamine Analogues

Quantitative structure-activity relationship (QSAR) has been used in drug-design studies since the 1960s (Martin 1978). Typically, a number of possible independent variables, usually physiochemical parameters relating to a series of compounds, are evaluated for correlation with activity values using multiple-regression analysis.

Previous studies have generated some relationship between the structural property and the biological activity of polyamine analogues, such as terminal alkylation and the required presence of positive charged nitrogens (Bergeron et al. 1994). Further studies are needed to explain the dramatic activity difference caused by small variations in structures. An obvious example is that although structurally DEHSPM, DESPM and DENSPM are different only in one or two methylene bonds, they show great difference in bioactivity and toxicity (Bergeron et al. 1989 and 1994). Based on a large number of polyamine analogs synthesized in our group and some from other researchers, a library of diverse analogues is established for this QSAR study. I believe that a computer-assisted QSAR study could serve as a guide to the mechanism of the drug action and could assist in the design of new analogues.

A comparative molecular field analysis (CoMFA) was considered as the approach to define overall QSAR (Plummer 1990). The idea underlying a CoMFA is that differences in a target property are often related to differences in the shapes of the non-covalent fields surrounding the tested molecules (SYBYL 1995). To put the shape of a molecule field into a QSAR table, the magnitudes of its steric (Lennard-Jones) and electrostatic fields (Coulombic) are sampled at regular intervals throughout a region. While there are many possible adjustable parameters in CoMFA, the most important is the relative alignment of the individual molecules when their fields are computed.

Method of Data Analysis

Partial least squares (PLS), in CoMFA, like multiple regression (MR) used in classical QSAR, is used to derive linear equations which describe the differences in the values of targeted properties based upon the differences in the values of their corresponding parameters (SYBYL 1995). As implemented in SYBYL/QSAR, PLS might be described as a major extension of MR. In any MR analyzed QSAR, there exists a potential error called chance correlation, a correlation which is only an accidental correspondence among numbers which in fact are not related. PLS could omit this risk and has less restrictions on the number of compounds and parameters compared to MR.

Methods of Analysis Validation

CoMFA uses several statistical tools to examine the accuracy and stability of the derived equation. They are crossvalidation and bootstrapping. Crossvalidation examines the accuracy of QSAR predictions. The crossvalidation technique involves random elimination of one or more analogues from the original data set with subsequent equation development and activity prediction for the eliminated analogues in an interactive manner. This develops a QSAR equation that is generally of greater predictive value than that derived from conventional regression analysis. The result of crossvalidation is reported in

the form of crossvalidated r^2. Crossvalidated r^2 is defined as the fraction of original "variance" (squared differences among target values) predicted by QSAR. The r^2 ranges from 1 to 0, sometimes include negative numbers. When $r^2 = 1$, a perfect model has been found. If $r^2 = 0$, then there is absolutely no relationship between structure and activity. Negative r^2 doesn't necessarily imply "no model" at all, but highlights that additional work is needed. In this type of study, when r^2 is greater than 0.4, useful and statistically significant results are implied.

Another modern validation method used in CoMFA is bootstrapping. Bootstrapping evaluates the stability of the equation derived by the regression analysis. The name is derived from the adage for self-advancement occurring when you pull yourself up by your own bootstraps. The idea is to simulate a statistical sampling procedure by assuming that the original data set is the true population and generating many new data sets from it. These new data sets (called bootstrap samplings) are of the same size as the original data. Repeated selection of the same row is allowed. The difference between the parameters calculated from the original data set and the average of the parameters calculated from multiple bootstrap sampling is a measure of the bias of the original calculation. The calculated variance of the parameter estimates reflects the accuracy with which any of the parameters can be estimated from the input data.

Computer Modeling

In early studies, only terminal alkylated tetraamines were included in the database (Bergeron et al. 1996b). A more accurate CoMFA can be obtained by expanding the database with more structurally diverse compounds. So, primary polyamines along with the relatively inactive polyamine derivatives reported before are included. In this study, a total of 99 polyamine analogues, including the triamine analogues synthesized in this study were used in the final database (Table 2-1). All of the polyamine analogues listed in Table

2-1 are numbered differently from the other chapters. In other chapters where compound numbering are referred (Chapter 3, 4, 5 and 7), the same numbering system is applied.

The biological activity parameter used in this QSAR study is K_i. The K_i values are the concentration of drugs required to inhibit the uptake of radioactive spermidine transport by 50%. The K_i values of diamines (**60-65**), triamines (**56-59**) and triamine analogues (**39**,**40**,**42-52**,**54**,**55**) and part of the tetraamine analogues (**2-5**, **7-11**,**14-37**) are from other studies (Porter and Bergeron 1983, Porter et al. 1985, Bergeron et al. 1994). While the K_i values of norspermidine, spermidine, homospermidine and spermine had been reported long time ago, the K_i values presented in this study were repeated as positive control, and most of them agree well with the previous values.

On the basis of pKa values measured (Bergeron et al. 1995a) for DENSPM (**4**), DESPM (**11**), DEHSPM (**17**), N^1,N^{12}-bis(2,2,2-trifluoroethyl)spermine (FDESPM **26**), PIP(4,4,4) [*N*,*N*'-bis(4-piperidinylmethyl)-1,4-diaminobutane (**32**)], PYR(4,4,4) [*N*,*N*'-bis(4-pyridylmethyl)-1,4-diaminobutane (**33**)] and PIP(5,4,5) [*N*,*N*'-bis{2-(4-pyridylethyl)}-1,4-diaminobutane (**35**)] (Table 2-1), the following assumption about the protonation state have been made. All of the nonaromatic, nontrifluoroethylated tetraamines are largely in the from of tetracation at physiological pH 7.2. The spermine analogues (**9-13**) and the homospermine analogues (**15-21**) and homologues (**22-25**) should be 85 and 97%, respectively, in the form of tetracations. The norspermine analogues (**2-7**) should at least 74% in the form of the tetracations. Compounds **26**, **28**, **30**, **33** and **35** should be almost exclusively in the form of internal dication at physiological pH. Compound **27** should also be a dication but because of the resonance it should be a terminal dication. Compound **31** should be a dication with N^1 and N^3 charged. All of the unacetylated diamines and triamines are treated as dications and trications, while the acetylated nitrogens are not charged. Thus the calculation of atomic charges and conformational search of the analogues are carried out based on the anticipated protonated structures. Two non-polyamines, N^4-benzylspermidine nitrile **98** and MGBG

99 are also included in the database for their ability to compete for polyamine transport systems (Porter et al. 1982).

The low energy conformations of polyamine analogues were obtained by systematic conformation searching supported by Tripos Associates, Inc. in SYBYL 6.2 molecular modeling program. The energies of steric and electrostatic interaction between each of the analogues were calculated as parameters. A probe atom was placed at the various interactions of regular three-dimensional lattice, which was large enough to include all of the analogues in the database and with a 2.0 Å lattice space. The probe atom had the shape of sp^3 carbon and a charge of +1.0. The Van der Waals values taken from the standard Tripos force field and the atomic charges were calculated by the method of Gastereiger and Marsili (Gastereiger & Marsili 1980, SYBYL 1995). Wherever the probe atom experiences a steric repulsion greater than "cutoff" (30 kcal/mol in these studies), the steric interaction is set to the value "cutoff", and the electrostatic interaction was set to the mean of the other molecular electrostatic interactions at the same location.

As mentioned before, the initial alignment of the molecules, the positioning of a molecular model within the fixed lattices, is by far the most critical step in developing a successful CoMFA model, since the relative interaction energies depend strongly on relative molecular positions. In this study, polyamines were aligned by using DESPM (**11**) as a template. In the tetraamines, both the end nitrogen atoms of the analogues and the third nitrogen atom were used as atom pairs between two molecules for performing a best fit. All of the triamines were aligned by using the three nitrogen atoms. Two nitrogen and one carbon atoms were used for molecular match in diamines. The resulting structures will be used to generate the CoMFA analysis.

The resulting database as described above with steric and electrostatic field values, on a regularly spaced lattice around the analogues, were then correlated with the K_i data for the polyamine transport system by a very efficient statistical method mentioned above, PLS (Cramer 1988). Statistical techniques, bootstrapping and crossvalidation, are used to

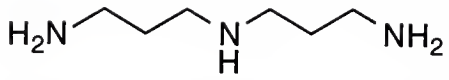
determine the quality of the correlation. The implementation of PLS also rotates the PLS solution back into the original data space, thus generating a "conventional" QSAR equation showing r-square, F test, and the standard error S.

Results

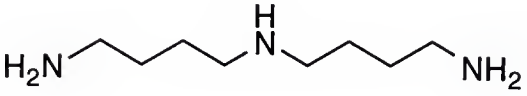
The QSAR CoMFA studies by using a total of 99 polyamine analogues provided a model with a crossvalidated $r^2 = 0.810$ (optimum component 5) and the conventional QSAR equation ($R^2 = 0.959$, F test = 431.506, S = 0.199). The molecular modeling shows that the relative contribution of the steric and electrostatic term in the QSAR equation are 0.708 and 0.292, respectively, supporting the importance of the geometry of the groups fixed to the nitrogen and of charge.

All of the polyamine analogues in the database, including diamines, triamines and tetraamine analogues are listed in Table 2-1. The calculated K_i values of polyamine analogues by the QSAR CoMFA equation, compared with the actual , are also listed (Table 2-1). The differences between the predicted and actual K_i values are generally small. Figure 2-3 graphically demonstrates the significant correlation that exists between the experimental versus the calculated K_i values.

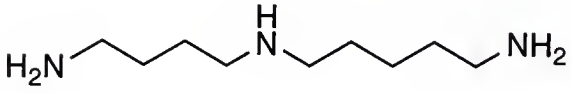
This CoMFA model of 99 compounds was generated using different kinds of structures of polyamine derivatives with linear, cyclic, and aromatic substituents. As the K_i values used in the model are widely distributed, from 0.7 to 4500 μM, it is expected that this CoMFA model would be very powerful in the evaluation of K_i values of new structures.



Norspermidine (3,3-Triamine)



Homospermidine (4,4-Triamine)



4,5-Triamine

Figure 2-1: Structure of unusual triamines found in plants and bacteria.

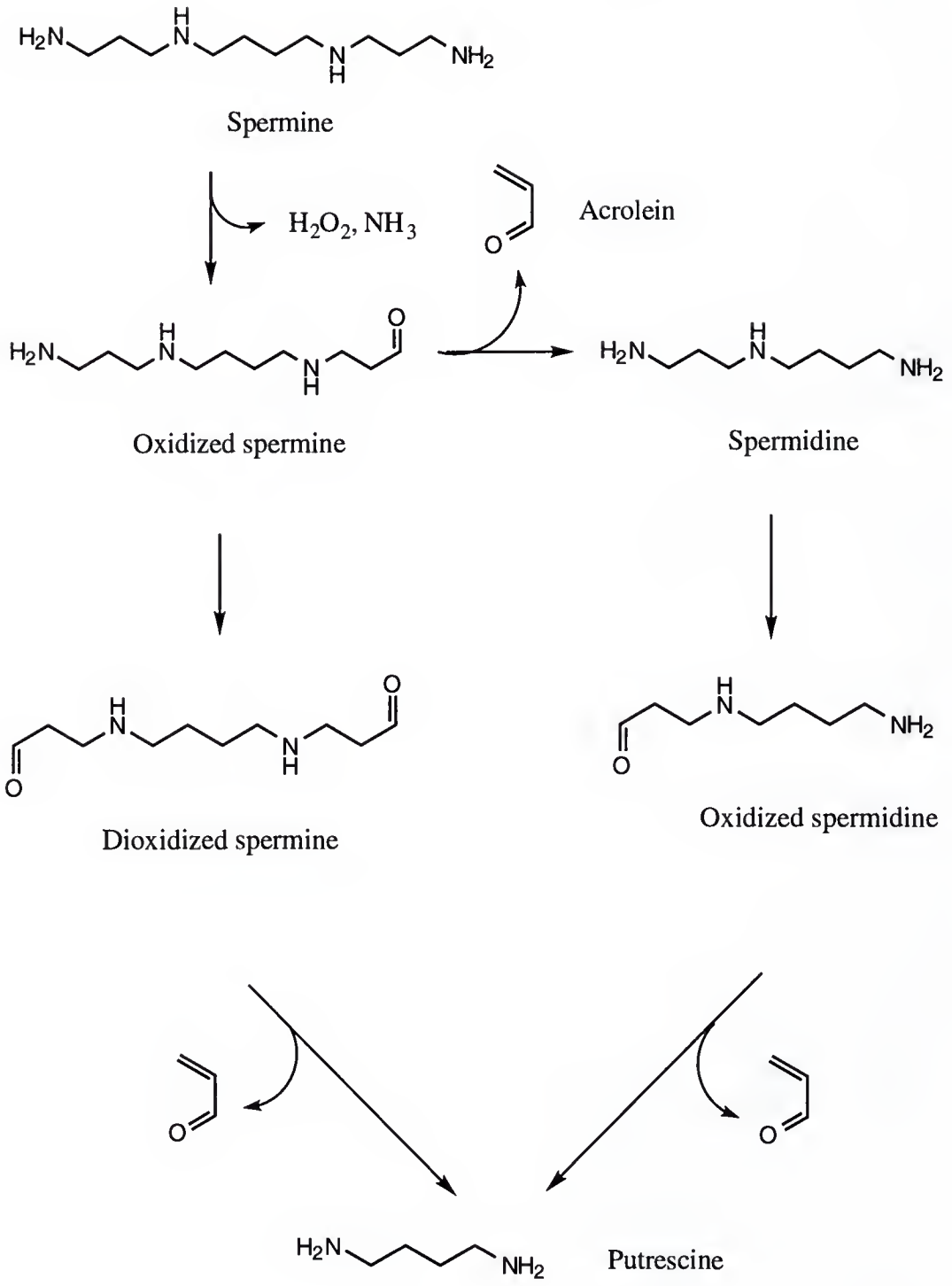


Figure 2-2: Oxidative deamination of spermidine and spermine by serum amine oxidase. Ammonia, hydrogenperoxide, acrolein and aldehydes were generated.

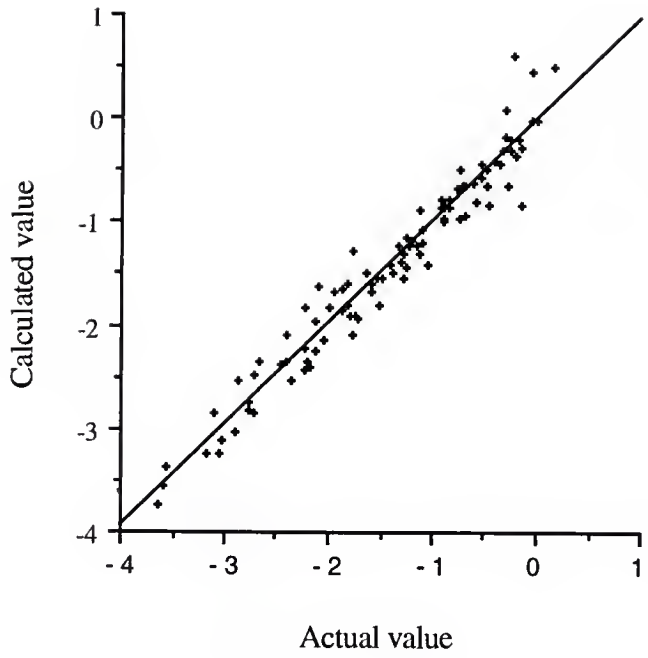


Figure 2-3: Measured versus calculated activity values for polyamine analogues. The values presented here were expressed in the form of $-\log K_i$, directly used in the molecular modeling. Each point represented a polyamine analogue, and 99 polyamine analogues were included in this database.

Table 2-1: Comparison of actual and calculated Ki values of polyamine analogues.

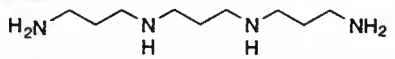
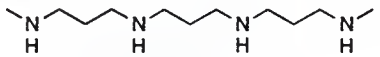
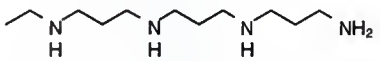
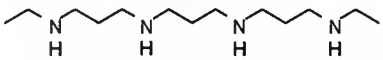
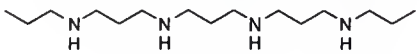
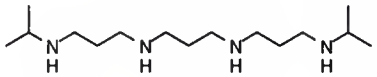
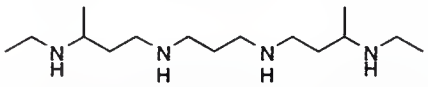
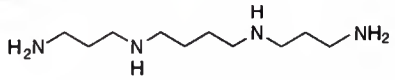
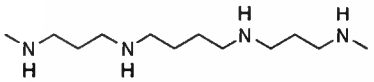
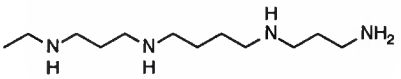
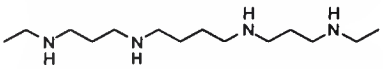
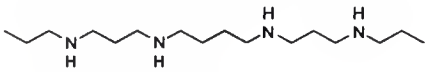
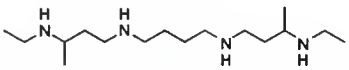
	Structure		K_i (μM)	
		Abbreviate	Actual	Calculated
Norspermines				
1		NSPM	4.2	4.1
2		DMNSPM	5.6	3.0
3		MENSPM	7.7	9.1
4		DENSPM	17	17
5		DPNSPM	11	26
6		DIPNSPM	40	39
7		DECroNSPM	97	69
Spermine				
8		SPM	0.7	0.9
9		DMSPM	1.1	1.0
10		MESPM	1.7	0.9
11		DESPM	1.6	2.3
12		DPSPM	2.3	2.7
13		DECroSPM	1.9	4.4

Table 2-1--Continuted

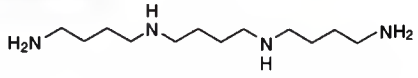
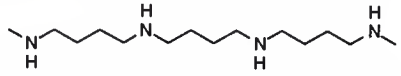
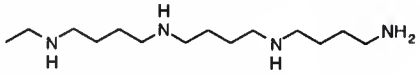
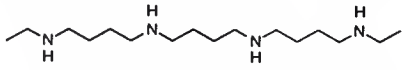
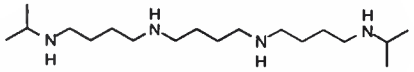
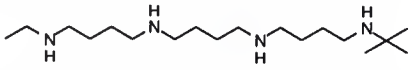
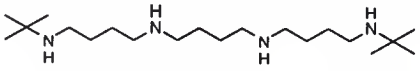
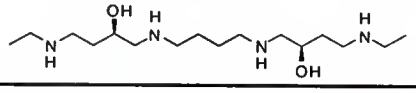
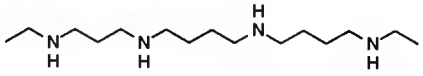
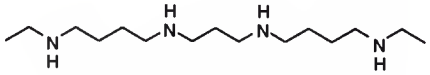
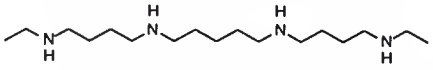
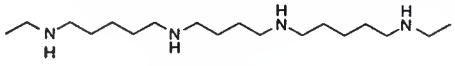
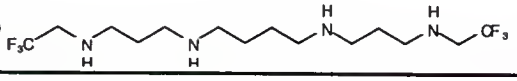
	Structure	Abbreviate	K_i (μM) Actual	Calculated
Homospermines				
14		HSPM	2.1	0.9
15		DMHSPM	0.97	1.1
16		MEHSPM	1.1	0.4
17		DEHSPM	1.4	1.9
18		DIPHSPM	8.1	9.8
19		ETBHSPM	3	3.1
20		DTBHSPM	56	79
21		(2OH)DEHSPM(R,R)	1.8	2.0
Homospermine Homologues				
22		DE(3,4,4)	8	7
23		DE(4,3,4)	4	6.5
24		DE(4,5,4)	6.0	4.8
25		DE(5,4,5)	16	15
26		FDES	285	245

Table 2-1--Continuted

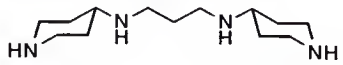
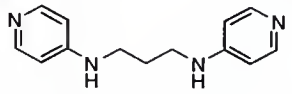
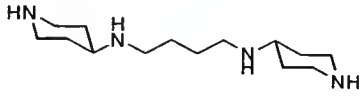
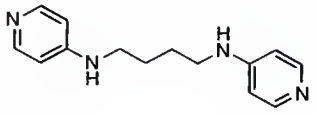
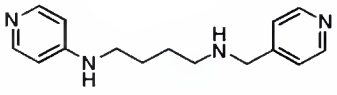
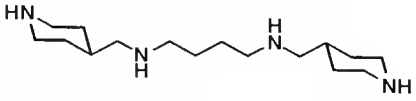
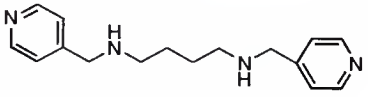
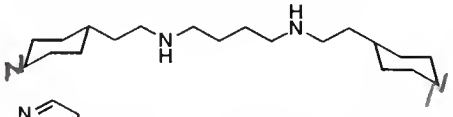
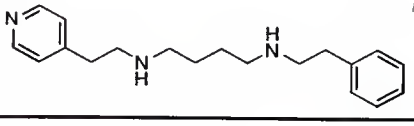
	Structure		K_i (μM)	
		Abbreviate	Actual	Calculated
Piperidine and Pyridine Derivatives				
27		PIP(3,3,3)	60.5	69.1
28		PYP(3,3,3)	3940	3548
29		PIP(3,4,3)	24.6	31.6
30		PYP(3,4,3)	4480	5495
31		PYP(3,4,4)	608	661
32		PIP(4,4,4)	4.9	4.4
33		PYP(4,4,4)	3750	2399
34		PIP(5,4,5)	18.1	19.1
35		PYP(5,4,5)	>500	1096

Table 2-1--continuted

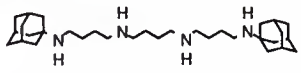
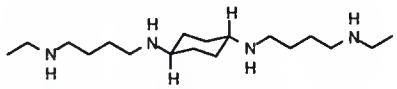
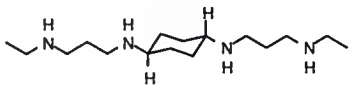
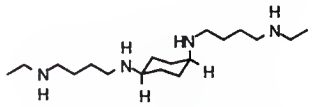
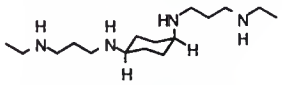
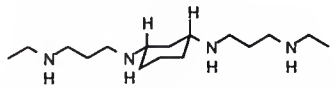
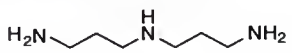
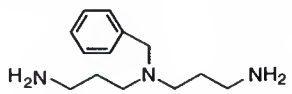
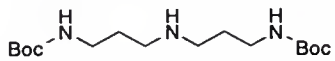
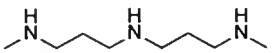
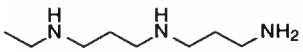
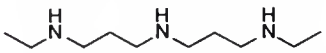
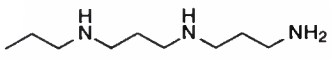
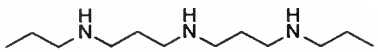
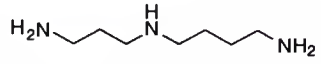
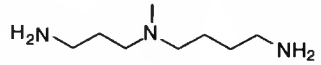
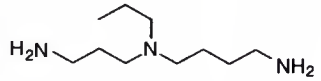
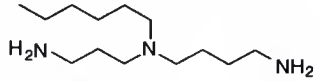
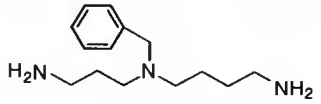
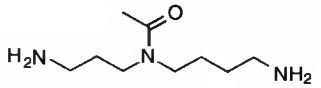
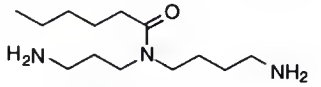
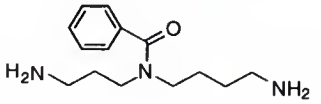
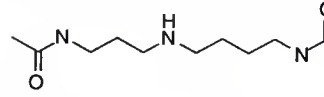
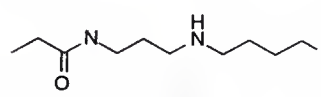
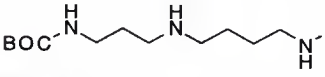
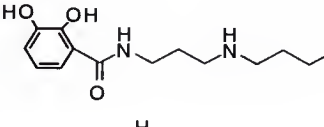
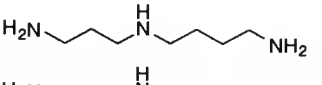
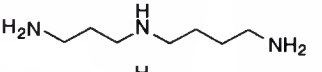
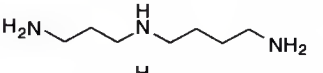
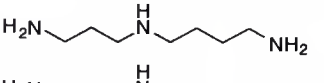
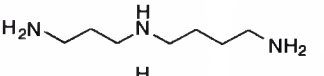
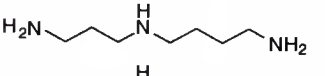
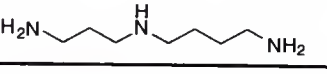
	Structure		K_i (μM)	
		Abbreviate	Actual	Calculated
Cyclohexane Derivatives				
36		BAHSPM	2	1.5
37		CHX(4,4,4)-trans	3.5	2.7
38		CHX(3,4,3)-trans	7.9	7.2
39		CHX(4,4,4)-cis	1.5	1.6
40		CHX(3,4,3)-cis	1.8	1.6
41		CHX(3,3,3)-cis	30	35
Norspermidine Analogues				
42		NSPD	7.2	7.2
43		N^4-Benzyl-NSPD	135	91
44		Bis(Boc)-NSPD	1100	1778
45		DMNSPD	60	19
46		MENSPD	34	34
47		DENSPD	250	123
48		MPNSPD	33	62
49		DPNSPD	125	42

Table 2-1--continued

	Structure		K_i (μM)	
		Abbreviate	Actual	Calculated
Spermidine Analogues				
50		SPD	2.2	2.0
51		N^4-Methyl-SPD	2.6	2.6
52		N^4-Propyl-SPD	3.1	4.5
53		N^4-Hexyl-SPD	43	32
54		N^4-Benzyl-SPD	39	47
55		N^4-Acetyl-SPD	115	135
56		N^4-Hexanoyl-SPD	151	257
57		N^4-Benzoyl-SPD	>500	269
58		Bis(acetyl)-SPD	>500	1259
59		Bis(propionyl)-SPD	>500	1698
60		Bis(BOC)-SPD	521	295
61		Bis(benzoyl)-SPD	256	229
62		DMSPD	5.1	4.5
63		MESPD(N1)	8.6	6.0
64		MESPD(N8)	7	6.0
65		DESPD	19.3	20.4
66		MPSPD(N1)	3.0	8.3
67		MPSPD(N8)	8.5	7.1
68		DPSPD	25.6	25.1

All wrong structures!

Table 2-1--continuted

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		Abbreviate	Actual	Calculated
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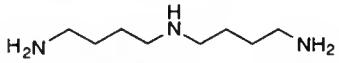
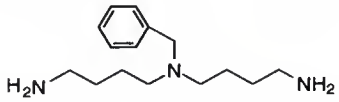
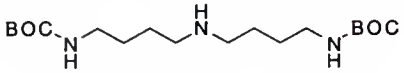
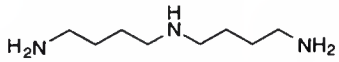
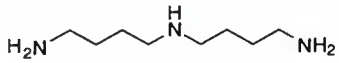
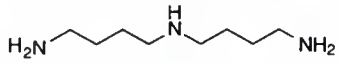
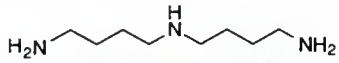
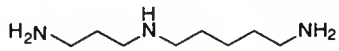
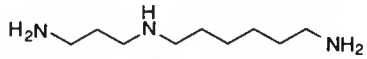
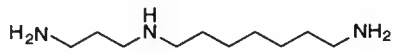
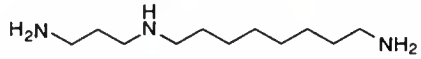
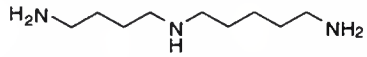
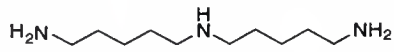
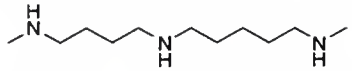
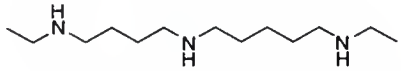
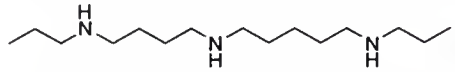
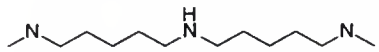
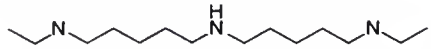
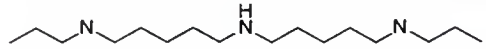
69		HSPD	3.4	3.6
70		N^4-Benzyl-HSPD	14	16
71		Bis(BOC)-HSPD	504	550
72		DMHSPD	5.5	9.1
73		DEHSPD	19	35
74		MPHSPD	5.0	8.5
75		DPHSPD	67	40
Spermidine Homologues				
76		Triamine(3,5)	12.3	11.5
77		Triamine(3,6)	13.1	7.6
78		Triamine(3,7)	13.0	15.8
79		Triamine(3.8)	7.8	7.2
80		Triamine(4,5)	1.4	6.6
81		Triamine(5,5)	13.8	20
82		DM(4,5)	21	24.6
83		DE(4,5)	64	63
84		DP(4,5)	75	44
85		DM(5,5)	133	178
86		DE(5,5)	174	178
87		DP(5,5)	87	46

Table 2-1--continuted

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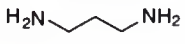
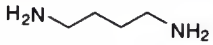
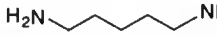
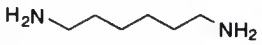
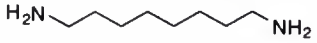
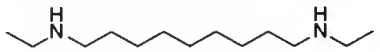
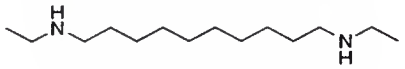
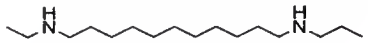
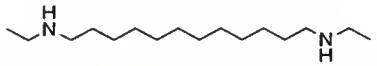
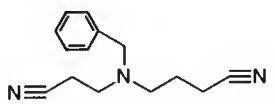
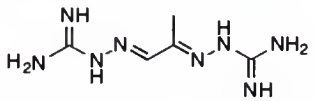
		Abbreviate	Actual	Calculated
Diamine Analogues				
88		DA_3	>500	72.4
89		DA_4	171	68
90		DA_5	459	229
91		DA_6	63	79
92		DA_7	18	27.5
93		DA_8	22	16.6
94		Et-9-Et	721	339
95		Et-10-Et	540	692
96		Et-11-Et	1230	692
97		Et-12-Et	232	347
Other Compounds				
98		N^4-Benzyl-SPD nitrile	163	219
99		MGBG	53	87

CHAPTER 3
SYNTHESIS

Previous Studies on Polyamine Synthesis

The early work on polyamine analogue synthesis started with the modification of linear methylene bridges between the nitrogens. A series of spermidine and spermine homologues with structure of $H_2N(CH_2)_x(CH_2)_2NH_2$ and $H_2N(CH_2)_3NH$-$(CH_2)_xNH(CH_2)_3NH_2$ (x = 2-6, 9, 10,12) were synthesized by mono or symmetrical dicyanoethylation of the appropriate α,ω-alkylene diamines, followed with catalytic reduction of nitriles (Israel 1964).

The modification of different nitrogens of polyamines was once to be the major tasks of chemists. Direct regioselective functionalization of nitrogens is difficult because the reactivity difference between primary and secondary amines are quite small. So, the synthetic strategy is to block the other nitrogens while leaving one free to be functionalized.

Functionalization with (*tert*-Butoxycarbonyl) Group

N^2,N^3-Diprotected spermidine was synthesized by masking N^2 and N^3 with *tert*-butoxycarbonyl (BOC) group (Humora & Quick 1979). Monodiaminonitrile obtained by the method of Israel was treated with 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON), a reagent previously utilized in peptide chemistry. The nitrile group was then reduced to amine by $LiAlH_4$ in a 70% yield. Then the N^1 nitrogen of spermidine could be functionalized and the BOC groups could be removed under acid condition, in this case, hydrogen chloride saturated methanol was used. This method can be applied not only to obtain a series of N^1-functionalized triamines, but also to reach the terminal functionalized spermine and its homologues.

Functionalization with Cycling Reagents

Another series of reagents offered functionalization at N^1 and N^8 of SPD by masking the spermidine as either cyclic urea or hexahydropyrimidine (Figure 3-2, McManis & Ganem 1980, Chantrapromma & Ganem 1981). The urea was produced in 95% yield from spermidine by exhaustive acylation ($ClCO_2CH_3$) and then hydrolyzed by $Ba(OH)_2$. One application of this intermediate is in a three-step synthesis of spermine from spermidine. Following the alkylation of cyclourea at N^8 (relative to spermine) with acrylonitrile, reduction and hydrolysis, spermine was released in a yield over 50%. The pyrimidine system could be functionalized and opened with ethyl hydrogen malonate and piperidine or pyridine. When regioselective acylations and alkylations reagents are available, N^1 or N^8-derivatives can be obtained.

The methodology was also extended to the symmetrical terminal modification of spermines (Figure 3-2). Treatment of spermine with aqueous formalin solution gave a crystalline bis-hexahydropyromidine in 95% yield. After acylation of the N^1 and N^{12} with the corresponding chloride, the gemdiamine heterocycles were removed by a Knoevenagel reaction (ethyl hydrogen malonate, piperidine, ethanol, heat) with a yield above 80%.

Benzyl group as a protecting group

The secondary nitrogen-modified spermindine and its analogs were synthesized by using benzylamine (**1**) as the starting material, which provides the central block--the secondary nitrogen of the triamines (Figure 3-3) (Bergeron 1986). In proceeding to the norspermidine reagent, benzylamine (**1**) can either be directly reacted with excess acrylonitrile under pressure (step a) to produce the bis(nitrile) (**2**) or can be reacted at room temperature with a mole of acrylonitrile (step d) to produce the mononitrile (**3**) which can be reacted at room temperature with a second mole of acrylonitrile (step e) to generate (**2**). The spermidine reagent (**4**) can be prepared by alkylating the mononitrile (**3**) with 4-

chlorobutyronitrile in butanol in the presence of sodium carbonate (step f). Synthesis of the homospermidine reagent involves bisalkylation of benzylamine (**1**) with 4-chlorobutyronitrile (step c) as before to produce nitrile (**5**). Each of the nitriles (**2**, **3**, **4**, **5**) gave a over 80% yield. Reduction of these nitriles with either lithium aluminum hydride or with W-2 Raney nickel in ethanol in the presence of sodium hydroxide (step d) produced the corresponding amines in a high yield about 70-90%. The Raney nickel procedure is found to be more effective in this case.

The secondary-N-benzylated reagents were used in the synthesis of a large number of terminally N-substituted triamines, both acylated and alkylated systems. i.e. N^1, N^8-diethyl (or dipropyl) spermidine and N^1, N^8-diacetyl (or dipropionyl) spermidine. The alkylated compounds were generated by reduction of the corresponding acyl compounds with lithium aluminum hydride. The N^4 (relative to sperimidine) benzyl group is removed by hydrogenolysis over palladium chloride at atmospheric pressure.

The first threefold protected spermine, namely N^3-benzyloxycarbonyl-N^1-phthaloyl-N^2-tosylspermine was made in eight steps (Figure 2-4 (A), Eugster 1978) Obtained from phthalamiding of 1,4-dibromobutane with potasium phthalimide (**2**) (step b), N-(4-bromobutyl)phthalimide (**3**) was treated with NaN_3 (step c) to give N-(4-azidobutyl)phthalimide (**4**). Followed the reduction of (**4**) in alkohol/HCl with 10% Pd/C (step d), the amine (**5**) was acylated with benzyl chloroformate (step e)to get diprotected diamine (**6**). The phthalimide was removed by hydrazinolysis (step f), and the resulting amine (**7**) was converted to diamide (**8**) with p-toluenesulfonychloride (step g). Alkylation of (**8**) with N-(3-bromopropyl)phthalimide in DMF/K_2CO_3 (step h) generated the final triprotected spermidine (**9**) with a yield of 38%. The protecting groups at N^1, N^2 and N^3 can be removed selectively by hydazineolysis (H_2NNH_2, EtOH, reflux), catalytic hydrogenolysis (Pt/C) and reductive treatment with sodium/liquid ammonia respectively.

The systemetic synthesis of norspermidine, spermidine and homospermidine triprotected analogues was introduced in 1984 (Bergeron et al.). It generated tris-protected

spermidine in five high-yield steps [Figure 3-4 (B)]. The synthesis of the polyamine reagents starts from the appropriate N-benzyl protected amine. Nitrile (**1**) is obtained in high yield from benzylamine alkylatedwith acrylonitrile (step a), and reduced to diamine (**2a**, n = 3) by Raney nickel (step c). With the presence of excess putrescine, the analog diamine (**2b**, n = 4) was obtained by monobenzylation of putrescine with benzaldehyde under reductive amination conditions (formic acid) in high yields (80%) (step b). The diamines (**2**) are further protected by reacting them with 1 equiv. of 2-[[(tert-butoxycarbonyl)oxy]-imino]-2-phenylacetonitrile (BOC-ON) at low temperature (0 °C) (step c), give mono-acylation regioselectively at the primary amine site, resulting in **3a** and **3b**. Cyanoethylation of (**3a**) with acrylonitrile (step d) gave the nitrile (**4a**) in quantitative yield. Alkylation of (**3a**) and (**3b**) with 4-chlorobutyronitrile (step e) gave the nitriles (**4b**) and (**4c**), respectively, also in high yield (95%). The nitriles were then reduced with Raney nickel (step f) and acylated with trifluoroacetic anhydride (step g) to give the desired triprotected spermidine and its analogues. The conditions for the selective deprotections are rather mild. The debenzylation of benzylspermidine required only mild hydrogenolysis conditions, removal of the BOC groups only need brief exposure to trifluoroacetic acid (TFA), and removal of the N-trifluoroacetyl group can be simply finished by refluxing the corresponding amide with methanolic sodium carbonate.

The introduction of the p-tolulenesulfonyl protecting group facilitated the synthesis of a series of terminal alkylated polyamine analogues (Bergeron et al. 1988). The N^1,N^{12} terminally alkylated spermine compounds were synthesized by first mono-sulfonating all of the spermine nitrogens with p-toluenesulfonyl chloride, leaving only the terminal nitrogens to be alkylated. The resulting sulfonamides were next treated with sodium hydride in dimethylformamide followed by excess alkylating agents, e.g. methyl iodide, ethyl iodide or n-propyl iodide. The sulfonamides can be unmasked by treatment with sodium in liquid ammonia for 12 h. One disadvantage of this reduction is that it produces various yields (30-70%) and some insoluble byproducts. Later on, the mesitylenesulfonyl group was

reported to be a better protecting group than p-tolulenesulfonyl (Bergeron 1994). The mesitylenesulfonyl groups could be cleanly removed under reductive conditions with 30% HBr in HOAc/PhOH. The deprotection gave a predictable yield of 70-80%.

In order to meet the need of synthesizing a series of asymmetrical tetraamine analogues, an asymmetrically protected diamine (**1**) was introduced (Figure 3-5). The protecting groups, a mesitylenesulfonyl at each end and a BOC, can be selectively removed. This triprotected diamine can be deprotonated on the monosubstituted end and alkylated with fragmenting reagent (**2**). After the BOC group selectively removed with trifluoroacetic acid (TFA), the terminal amide can be alkylated with another polyamine fragmenting reagent (**3**). The resulting tetrasulfonamide (**4**) can be demasked with the method of HBr/HOAc as mentioned above to provide the corresponding tetraamine analogues.

Synthetic Methods

Synthesis of Tetraamines

Three different families of tetraamines were synthesized (1) those with linear methylene backbones and symmetrical alkyl groups at each terminal nitrogen, (2) those with linear methylene backbones but only one subsititution at one end, (3) those with symmetric but branch methylene backbones. The linear polyamines dimethyl-(**1**) and monoethyl-(**2**) norspermine and dimethylspermine **7** were accessed by alkylation of the corresponding tetrasulfonamide dianion (Figure 3-6, step b). However, linear symmetric tetraamine diisopropylnorspermine **5** as well as the dimethyl-(**12**) and diisopropyl-(**15**) homospermine were accessed via the "fragment synthesis" (Figure 3-6). In this case, the central diamine segment as its N,N'-bis-sulfonamide **51-53** was alkylated at each end with the appropriate fragmenting reagent N-(ω-halomethylene)-N-alkanesulfonamide (Figure 3-6, step d). This less direct method was empolyeed for these compounds because isopropyl bromide can not be used to alkylate tetrasulfoamide successfully, and homospermine along

with its homologue 1,6,12,17-tetraazaheptadecane (4,5,4-tetraamine) are not commercially available. The unsymmetric linear tetraamine, monoethylnorspermine **2**, was obtained by monoalkylation of tetrasulfonylnorspermine dianion **49** (Figure 3-6, step b).

The synthesis of polyamine-segmenting reagents **65-71**, Figure 3-7, began by reacting a primary amine, even if hindered, with methylenesulfonyl chloride (step a). *N*-Alkylsulfonamides **61-64** were deprotonated (NaH/DMF) followed by alkylation with the appropriate dihalide in excess to generate synthons **65-67** (step b). Thus *N*-(ω-halomethylene)-*N*-alkylsulfonamides of any length can be prepared. These polyamine synthons eliminate limitations associated with the availability of the starting tetraamines. In addition, while earlier methods were limited to accessing polyamine analogues with terminal primary alkyl groups, this approach allows for fixing primary, secondary, and tertiary alkyl groups to the terminal amines of polyamines.

In this study, an entirely new synthetic method was introduced to access the symmetric tetraamines with branching methylene backbones, diethylcrotyl-norspermine (DECroNSPM **6**) and -spermine (DECroSPM **11**) (Figure 3-8). The tetraamine backbones were obtained by fixing the protected branching segments **74** symmetrically to both ends of 3 or 4 carbon bridges provided by α,ω-dibromides. The synthesis of this segment began with crotononitrile (mixture of cis and trans), which reacted with excess amount of ethylamine in 50% NaOH aqueous solution (step a). The obtained ethylamino nitrile **72** was reduced to **73** with Raney nickel in methanolic ammonia (step b), and masked with mesitylenesulfonyl chloride to give disulfonamide **74** (step c). Alkylation of 2 equivalent of **74** with 1 equivalent of the appropriate α,ω-dibromide provided the tetrasulfonamide **75** or **76** (step d), which was then deprotected by 30% HBr in HOAc with presence of phenol to generate DECroNSPM (**6**) and DECroSPM (**11**), respectively (step e).

Synthesis of Triamines

Two different families of triamines were synthesized (1) those with symmetrical methylene backbones, with one alkyl group at a terminal nitrogen or an alkyl group at

both nitrogens, (2) those with unsymmetrical methylene backbones, with a single alkyl group at a terminal nitrogen or an alkyl group at both nitrogens. These include norspermidines, spermidines, homospermidines, (4,5) and (5,5) triamines, the numbers referring to the number of methylenes seperating the nitrogens. In the case of the N^aN^w-disubstituted norspermine and spermidine analogues the commercially available triamines, norspermidine [NSPD (**22**)] and spermidine ([SPD (**28**)], were sulfonated by mesitylenesulfonyl chloride (3 equiv.) under biphasic conditions (CH_2Cl_2/dilute NaOH) (Figure 3-9, step i). Triprotected amines **85** and **86** were then converted to their corresponding dianions (NaH/DMF) and alkylated with an excess of the appropriate primary alkyl idodine (step f). Finally the mesitylenesulfonyl blocking groups were cleanly removed under reductive conditions utilizing 30% HBr in HOAc and phenol to give terminal dimethyl-(**23**) and dipropyl-(**27**) analogues of NSPD and dimethylspermidine (**29**), which were isolated as their trihydrochloride salts (step g).

In order to prepare the symmetrically dialkylated derivatives of 4,4- (m = 2, n = 2) and 5,5-(m = 3, n = 3) triamines (Figure 3-9), mesitylenesulfonamide **77** was first dialkylated with 2 equivalents of either 4-bromobutyronitrile or 5-bromovaleronitrile in the presence of NaH to provide the dinitriles **79** and **81**, respectively (step h). The cyano groups were then hydrogenated with Raney nickel in methanolic ammonia (step d). The resulting primary diamines **82** or **84** were converted to their corresponding mesitylenesulfonamides **87** and **89** (step e). After alkylated with the appropriate primary halide (step f) and the HBr-promoted deprotection (step g), the dimethyl-(**37**,**46**) and dipropyl-(**40**,**48**) series of 4,4-, 5,5-triamines and diethyl 5,5-triamine [DE(5,5) (**47**)] were obtained.

Diethyl homospermidine [DEHSPD (**38**)] was made following another synthetic route (Figure 3-9). Alkylation of mesitylenesulfonamide **77** with two equivalents of *N*-(4-bromobutyl)-*N*-ethylmesitylenesulfonamide **66** (step c) resulted the triprotected HSPD analogue **95**, which was then deprotected with HBr/HOAc to give DEHSPD **38** (step g).

The diethylatd unsymmetrical 4,5-triamine analogue, DE(4,5) (**22**), was produced from *N*-(*tert*-butoxy)-*N*-mesitylenesulfonamide **102**, a diprotected ammonia synthon (Figure 3-10). This reagent was first alkylated with *N*-(5-bromopentyl)-*N*'-ethylsulfonamide **66** (step b) to give triprotected diamine **103**. The BOC group of **103** was then removed with TFA (step c), and the obtained disulfonamide **104** was alkylated with *N*-(4-bromobutyl)-*N*'-ethylsulfonamide **67** (step d) to complete the triamine framework. Unmasking of the amino groups of **105** with HBr led to the diethylated analogue of the 4,5-triamine [DE(4,5) (**43**)] (step e).

The other two dialkylated 4,5-triamines, DM(4,5) (**42**) and DP(4,5) (**44**), were produced by another route, Figure 3-9. Two consecutive monoalkylations of mesitylenesulfonamide **77** with 4-bromobutyronitrile (step a) and 5-bromovaleronitrile (step b) generated dinitrile **80** . The cyano groups of **80** were reduced with Raney nickel in methanolic ammonia, resulting in primary amine **83** (step d). Treatment of **83** with 2 equivalents of mesitylenesulfonyl chloride (step e), terminal dialkylation with iodomethane or 1-iodopropane (step f), and then unmasking of the amino groups led to DM(4,5) (**42**) and DP(4,5) (**44**) (step g), respectively.

Monopropyl norspermidine, MPNSPD (**26**), can be made directly by mono alkylating trimesitylenesulfonyl NSPD **85** (step f), derived from commercially available NSPD (**22**) (step i). However, synthesis of the SPD and HSPD monopropyl analogues, involves a "fragment synthesis" (Figure 3-11), because the asymmetric backbone of SPD makes the direct alkylation poorly selective and HSPD is not commercially available. The bulky triphenylmethyl group can only be introduced selectively at one of the terminal amino groups of either diaminopropane or diaminobutane (Figure 3-11). Triphenylmethyl chloride was stirred at room temperature with either diamine in a 5-fold molar excess (step a). The primary nitrogen of **106** or **107** was selectively sulfonated (step b) and alkylated with the appropriate segmenting polyamine segment reagent **68** or **69** (step c). The alkylation at this anion was highly selective. All of the protecting groups can be removed

with HBr in HOAc with presence of phenol (step e) resulting in N^1-monopropyl spermidine [MPSPD(N^1), (**33**)], N^8-monopropyl spermidine [MPSPD(N^8), (**34**)], and N^1-monopropyl homospermidine [MPHSPD, (**39**)].

Homospermidine and 4,5-triamine has been previously obtained by nonspecific alkylation of diamines with 2 equivalents of ω-bromoalkylphthalimide, followed by hydrolysis with 12 M HCl (Figure 3-12 (A), Okada et al. 1979). The obtained mixture of diamine, triamine and tetraamine were purified on a ion-exchange column to give a yield of 37% for HSPD. While the detailed synthesis of 4,5 triamine was not mentioned and its yield was not specified in the study, it is assumed that 4,5 triamine was obtained by an further alkylation of N-butylphthalimide diamine with 5-bromopentaphthalimide. Clearly, direct alkyation of diamines dose not provide easy access to triamines.

A better synthetic route was introduced to get HSPD in the form of triacetate salt at high yield (Figure 3-12 (B), Bergeron et al. 1981). The 5-benzyldinitrile was obtained from dialkylation of benzylamine with 4-chlorobutanenitrile under vigorous condition--115 °C over 20 h. Treatment of dinitrile with platinum oxide in glacial acetic acid under a hydrogen atmosphere gave HSPD triacetate in a yield over 85%.

In this study, an alternative synthetic route was chosen for its mild condition, easy workup and high yield (Figure 3-13). In this method, use of the aromatic imide to protect the primary amines avoided the solubility problems during attempted hydrogenation (Raney nickel, methanolic NH_3) of bis(3-cyanopropyl)mesitylenesulfonamide **82** (Figure 3-9). The *N*,*N*'-di(butylamino)mesitylenesulfonamide **113** was synthesized by dialkylation of anionized mesitylenesulfonamide **77** with 2 equivalents of 5-chlorobutylphthalimide in DMF (Figure 3-13, step a), followed by hydrazinolysis (step b) to give **82**. The final treatment of sulfonamide **82** with 30% HBr in HOAc released HSPD **36** in good yield (step c).

The synthesis of the parent 4,5- (**41**) and 5,5-(**45**) triamines involved hydrogenation (Raney nickel, methanolic NH_3) of the corresponding dinitriles without

solubility problems (Figure 3-9). Hydrogenation of the *N*-4-cyano-*N'*-5-cyano-sulfonamide **80** (Figure 3-9, step d), followed by unmasking of sulfonyl group with HBr/HOAc (step j) has produced 4,5-triamine (**41**). The 5,5-triamine (**45**) was synthesized in a similar manner by reduction of the symmetrical dinitrile **81** (step d), followed by treatment with 30% HBr in HOAc gave 5,5-triamine (**45**) in good yield (Figure 3-9, step j).

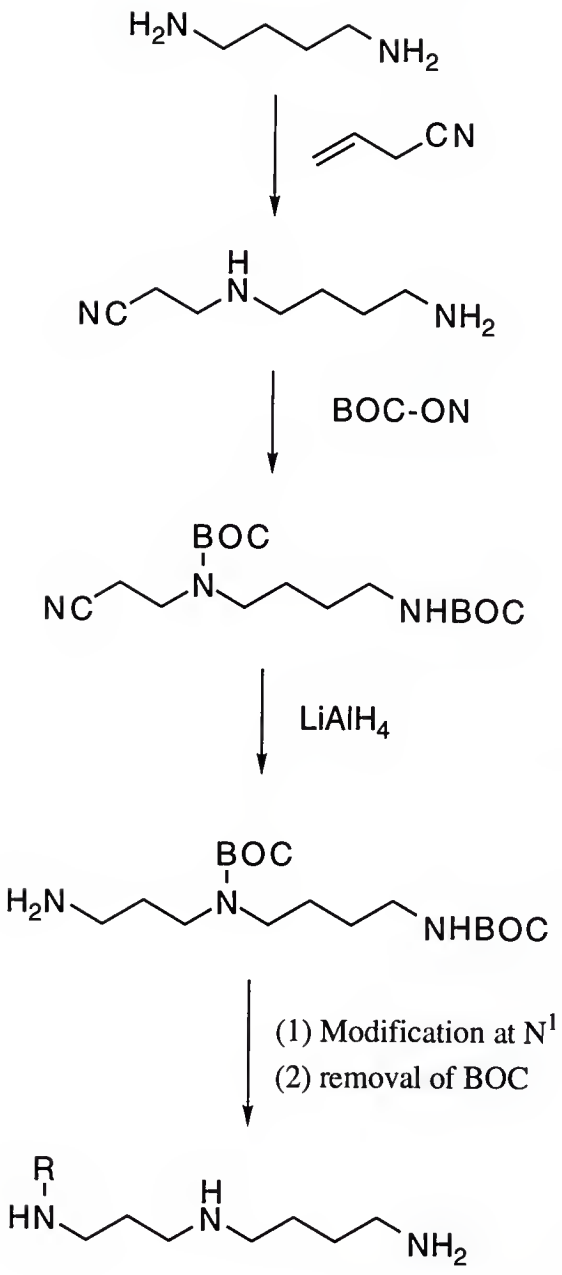


Figure 3-1: Humora and Quick's modification of spermidine at N^1 with *tert*-butoxycarbonyl group (BOC) as the protecting group.

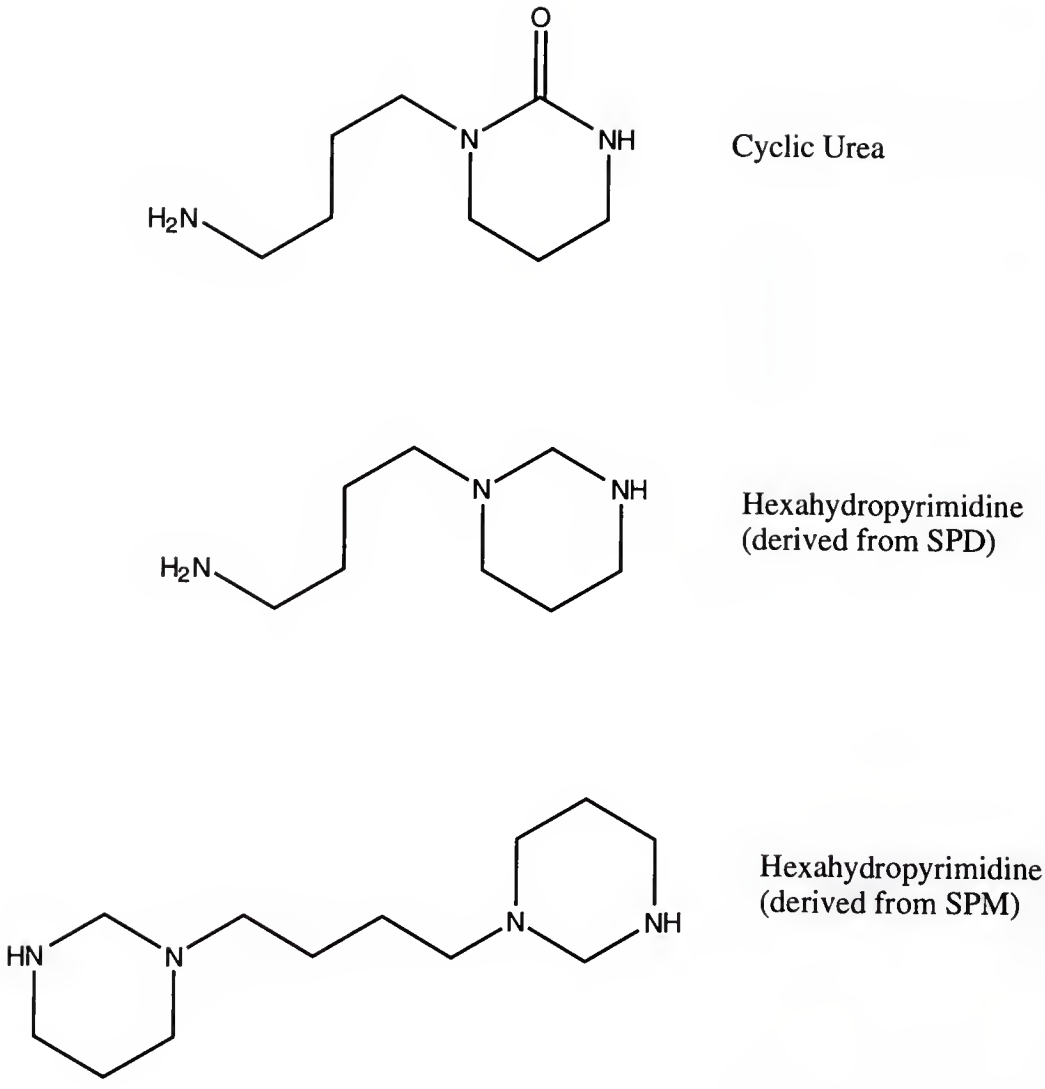


Figure 3-2: Ganem's masked polyamines as cyclic urea or hexahydropyrimidines.

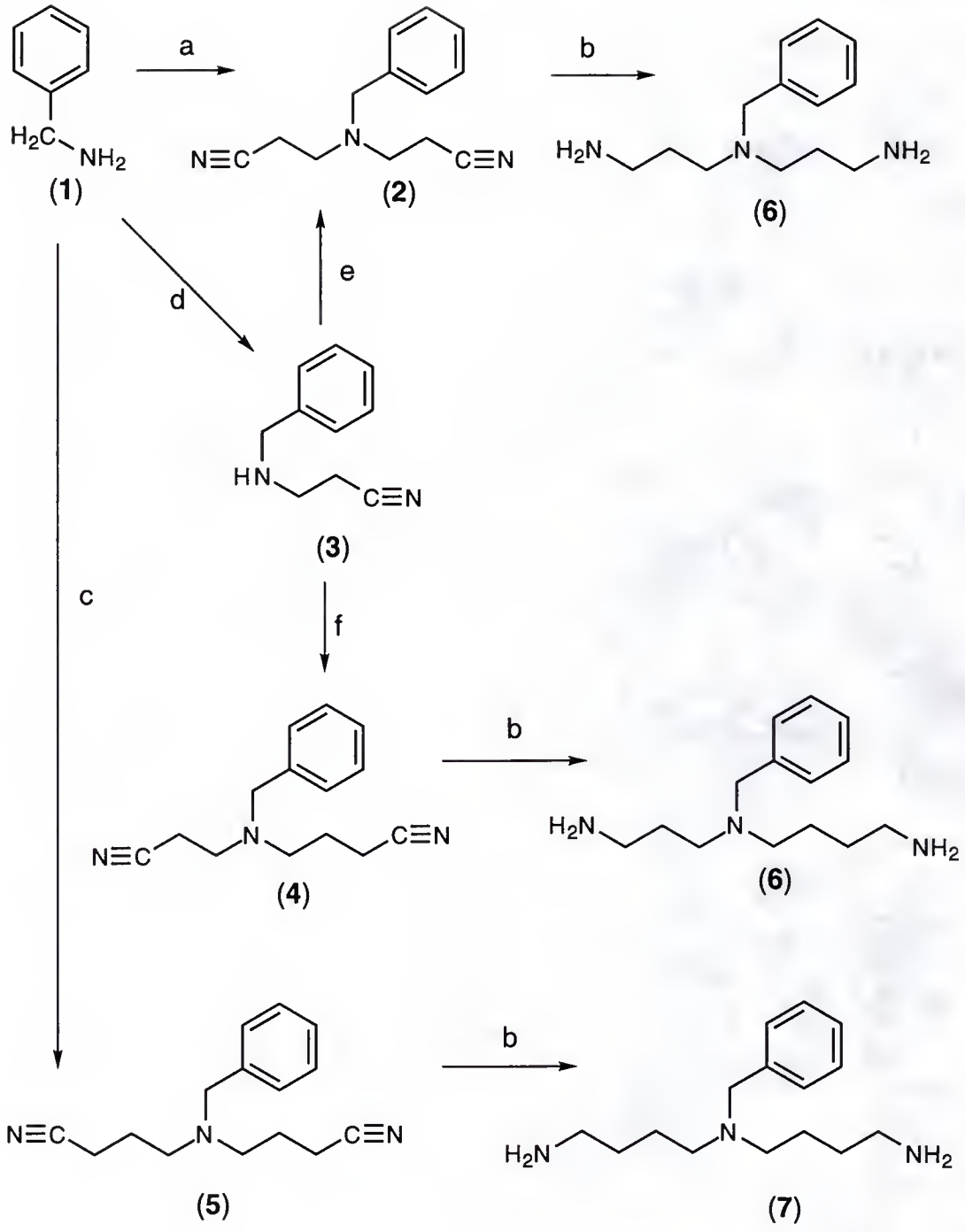


Figure 3-3: Modification of spermidine and its homologues at the secondary nitrogen. (a) Acrylonitrile (excess); (b) H_2/W-2 Raney nickel/NaOH/EtOH or $LiAlH_4$; (c) (f) 4-Chlorobutyronitrile/butanol/Na_2CO_3; (d) (e) Acrylonitrile (1 equiv.).

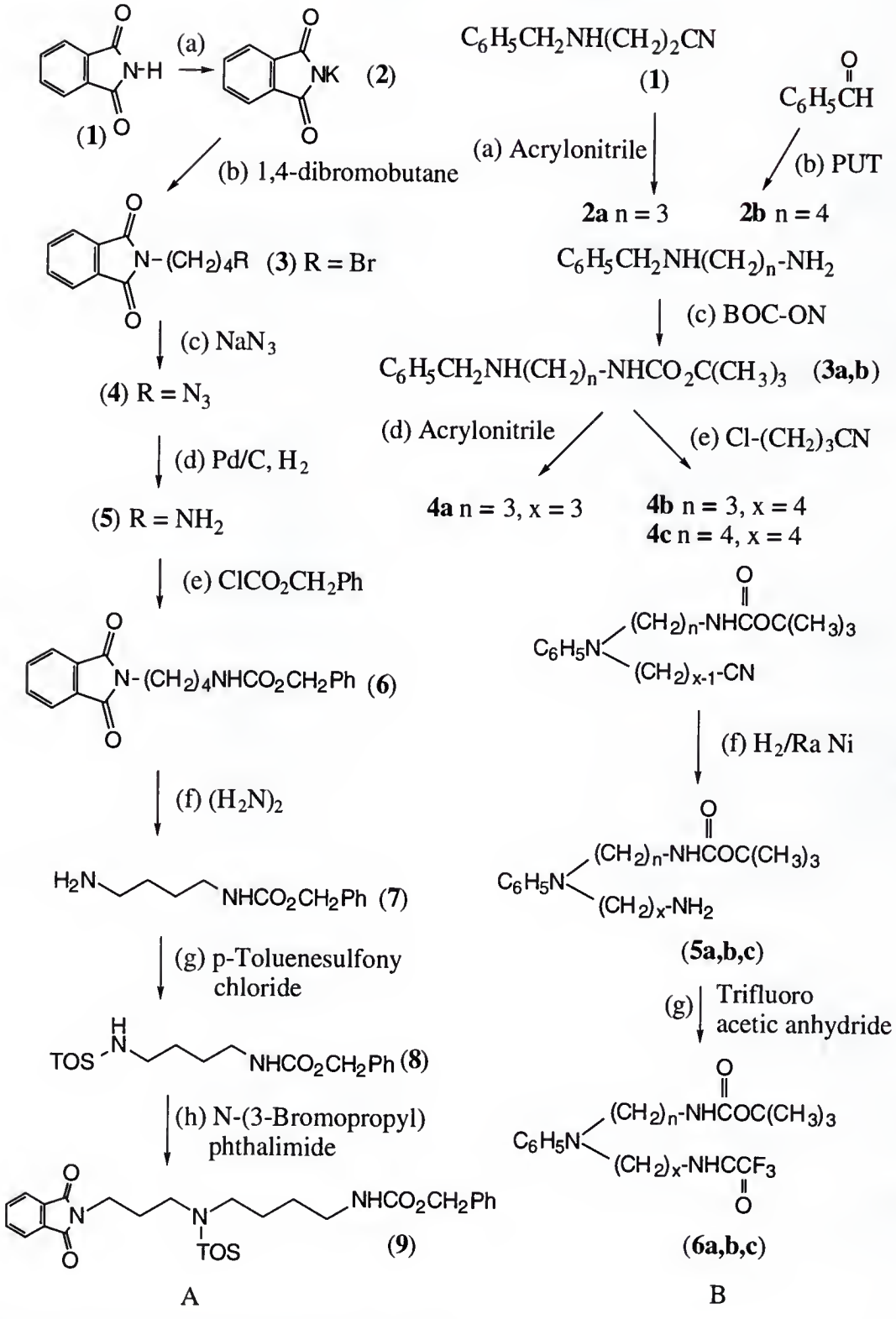


Figure 3-4: Synthesis of triprotected triamines.
(A) Eugster's eight-step synthesis of triprotected SPD. TOS = tosyl.
(B) Bergeron's five-step synthesis of triprotected NSPD (n = 3, x = 3); SPD (n = 3, x = 4) and HSPD (n = 4, x = 4).

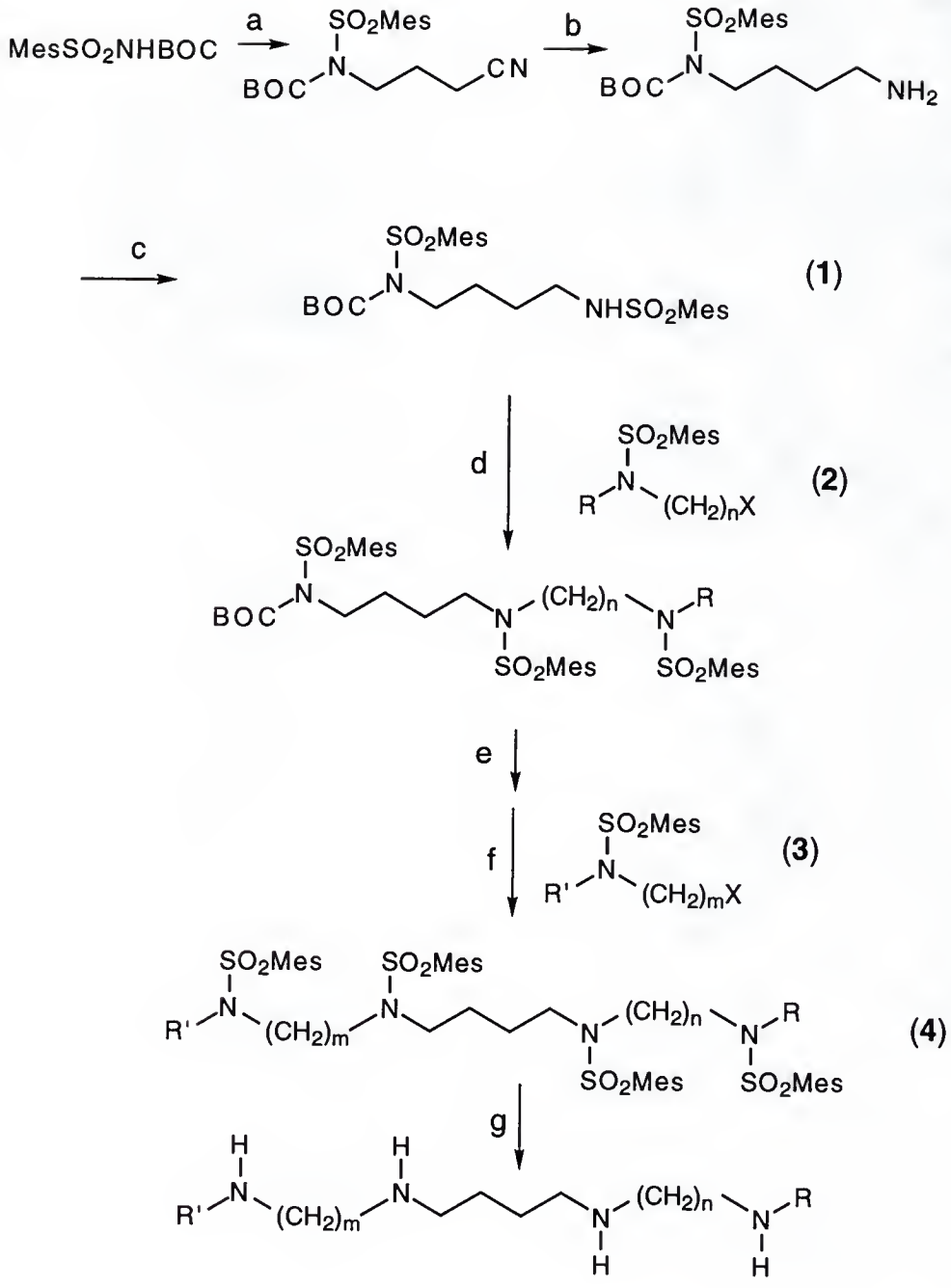


Figure 3-5: Synthesis of triprotected putrescine reagent and its application in synthesis of asymmetric spermine homologues. X = Cl or Br.
(a) 4-Chlorobutyronitrile/NaH/DMF; (b) H_2/Ra Ni/NH_3/CH_3OH;
(c) Mesitylenesulfonyl chloride/NaOH(aq)/CH_2Cl_2; (d) NaH/DMF/**2**;
(e) TFA/CH_2Cl_2; (f) NaH/DMF/**3**; (g) HBr in HOAc (30%)/PhOH, HCl.

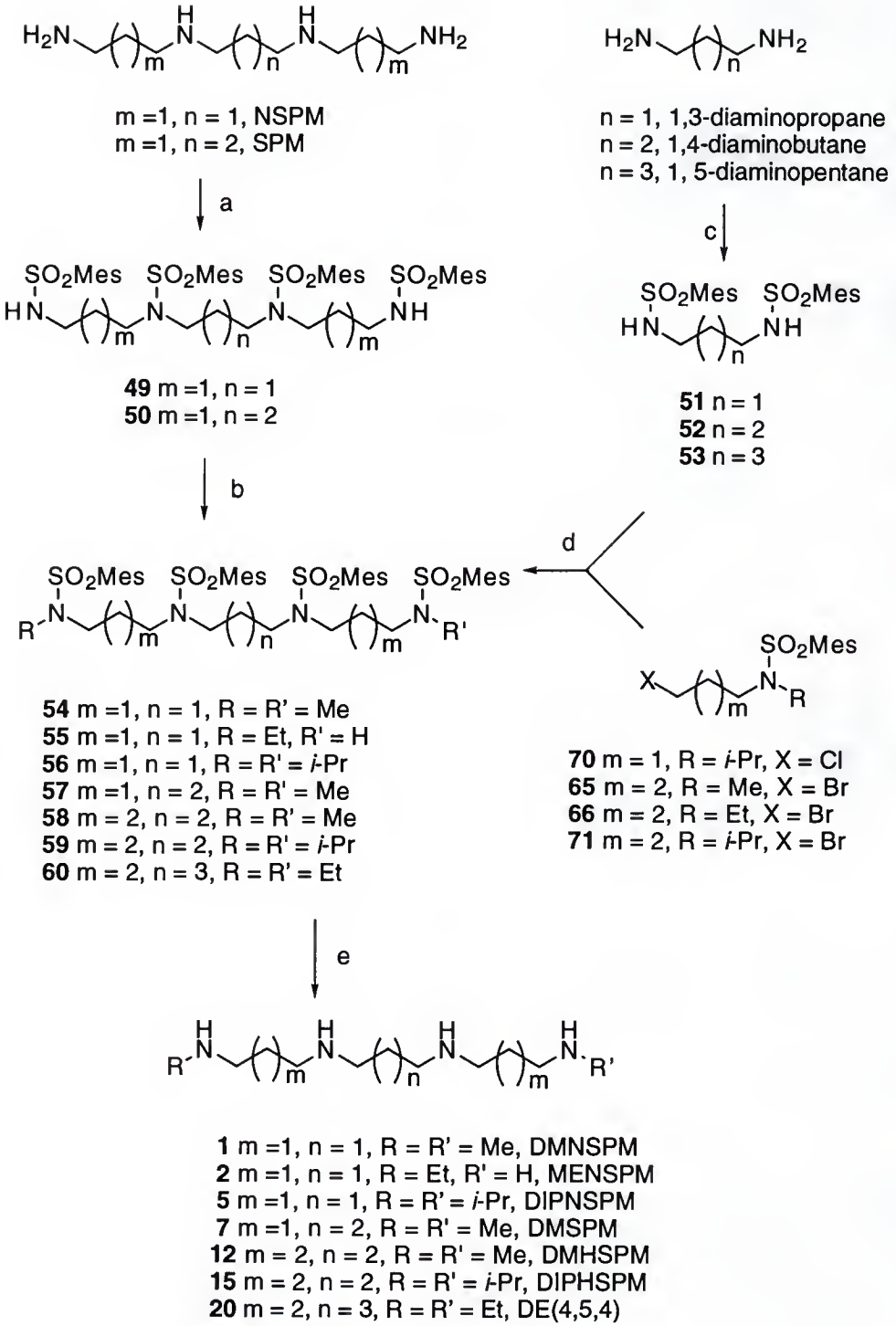
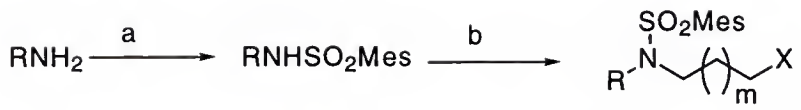


Figure 3-6: Synthesis of linear tetraamine analogues.
(a) (c) Mesitylenesulfonyl chloride/1 N NaOH(aq)/CH_2Cl_2;
(b) NaH/DMF/iodomethane or iodoethane; (d) NaH/DMF/ω-haloalkyl-sulfonamide (**65**, **66**, **70** or **71**); (e) HBr in HOAc(30%)/PhOH/CH_2Cl_2, then HCl.



61 R = Me

62 R = Et

63 R = *i*-Pr

64 R = n-Pr

65 R = Me, m = 2, X = Br

66 R = Et, m = 2, X = Br

67 R = Et, m = 3, X = Br

68 R = n-Pr, m =1, X = Br

69 R = n-Pr, m = 2, X = Br

70 R = *i*-Pr, m = 1, X = Cl

71 R = *i*-Pr, m = 2, X = Br

Figure 3-7: Synthesis of polyamine segmenting reagents.
(a) Mesitylenesulfonyl chloride/NaOH(aq)/CH_2Cl_2;
(b) NaH/DMF/α,ω-dihaloalkane.

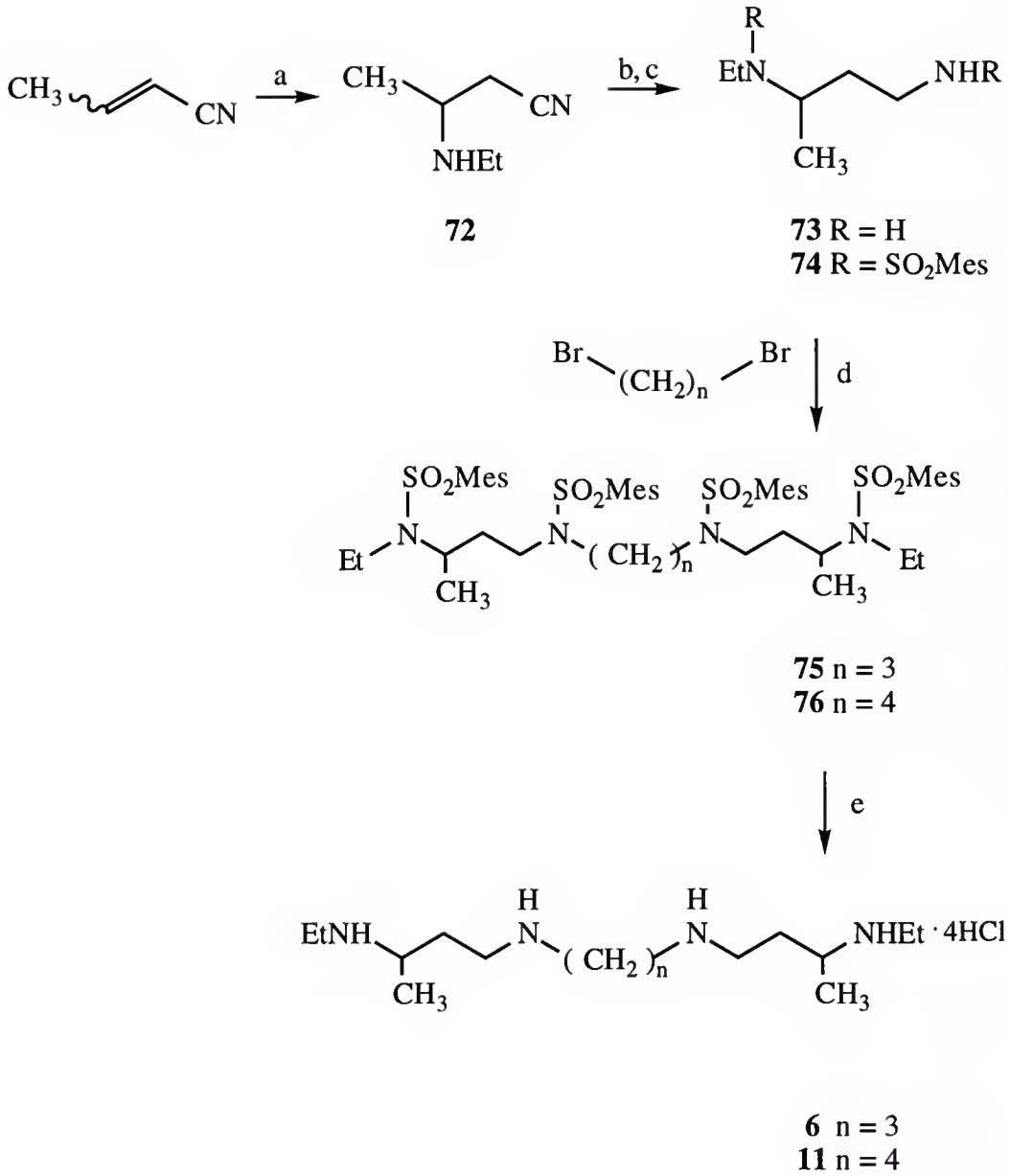


Figure 3-8: Synthesis of DECroNSPM and DECroSPM.
(a) $EtNH_2$/50% NaOH/CH_2Cl_2; (b) H_2/Ra Ni/NH_3/CH_3OH;
(c) Mesitylenesulfonyl chloride/NaOH(aq)/CH_2Cl_2; (d) NaH/DMF/
α,ω-dibromoalkane; (e) 30% HBr in HOAc/PhOH/CH_2Cl_2, then HCl.

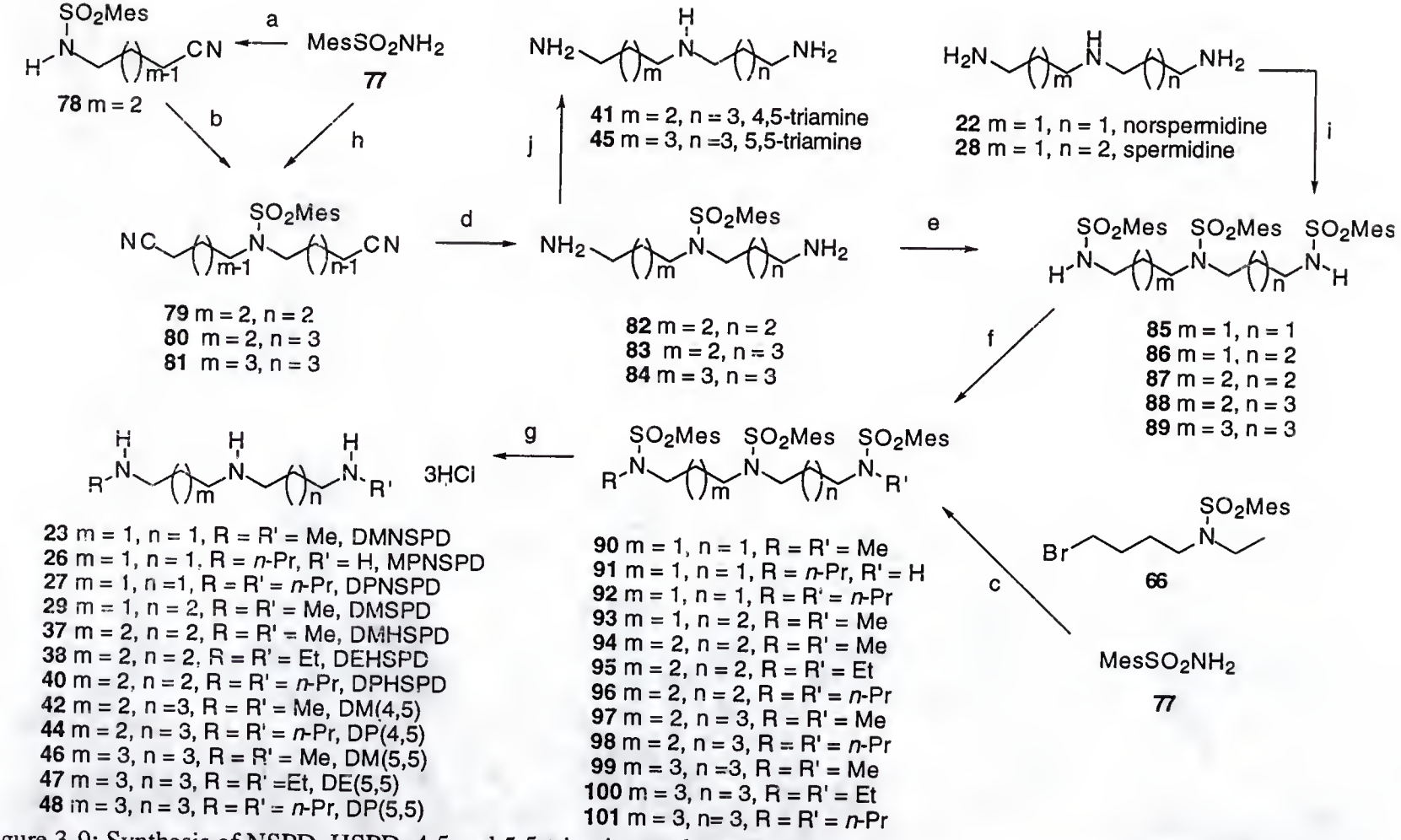


Figure 3-9: Synthesis of NSPD, HSPD, 4,5 and 5,5-triamine analogues.
(a) 4-Bromobutyronitrile(1 equiv)/NaH/DMF; (b) 5-Bromovaleronitrile(1 equiv)/NaH/DMF; (c) NaH/DMF; (d) H_2/Ra Ni/NH_3/CH_3OH; (e) Mesitylenesulfonyl chloride/NaOH(aq)/CH_2Cl_2; (f) NaH/DMF/haloalkane; (g) 30% HBr in HOAc/PhOH, HCl; (h) NaH/DMF/4-bromobutyronitrile (2 equiv) or 5-bromovaleronitrile (2 equiv); (i) same as (e); (j) same as (g).

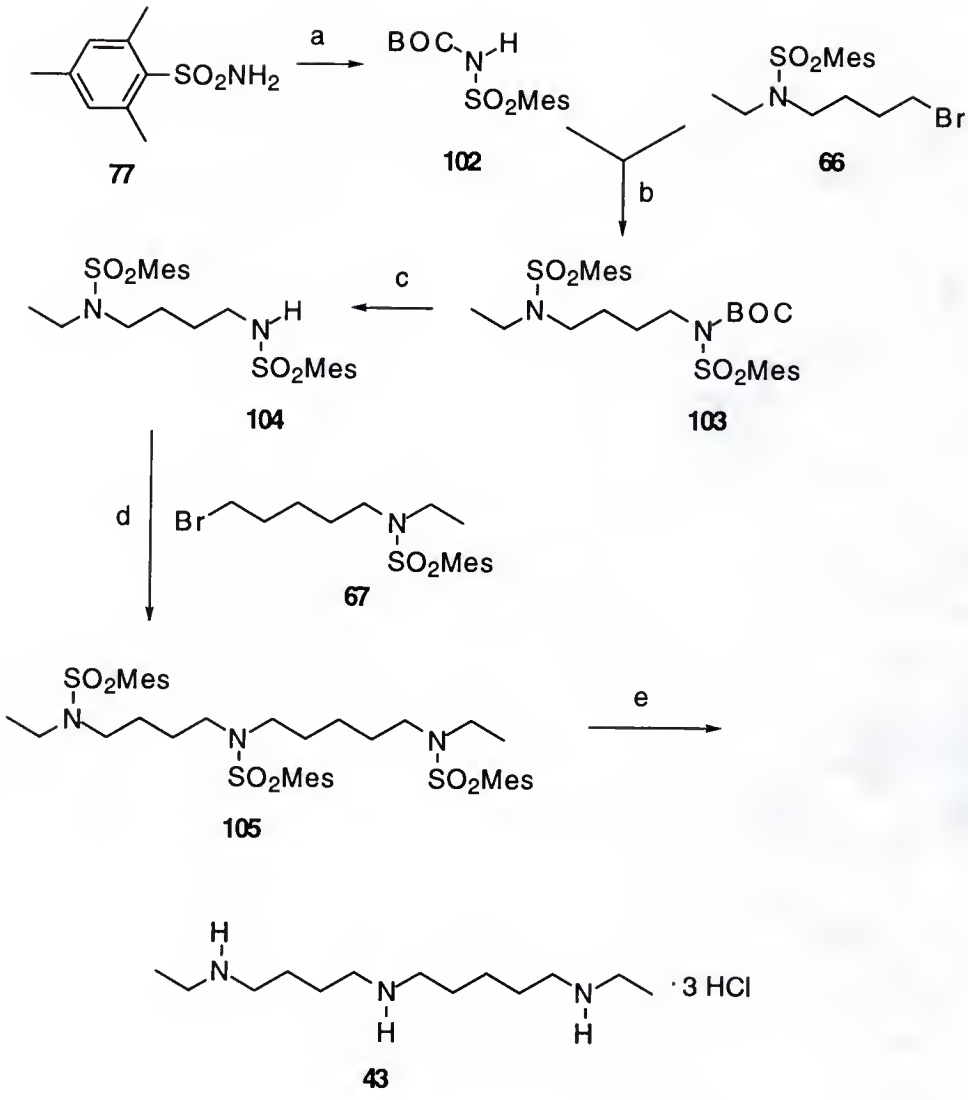


Figure 3-10: Synthesis of DE(4,5).
(a) Oxalyl chloride/*tert*-butyl alcohol; (b) NaH/DMF/**66**; (c) TFA/CH_2Cl_2; (d) NaH/DMF/**67**; (e) 30% HBr/HOAc/PhOH, then HCl.

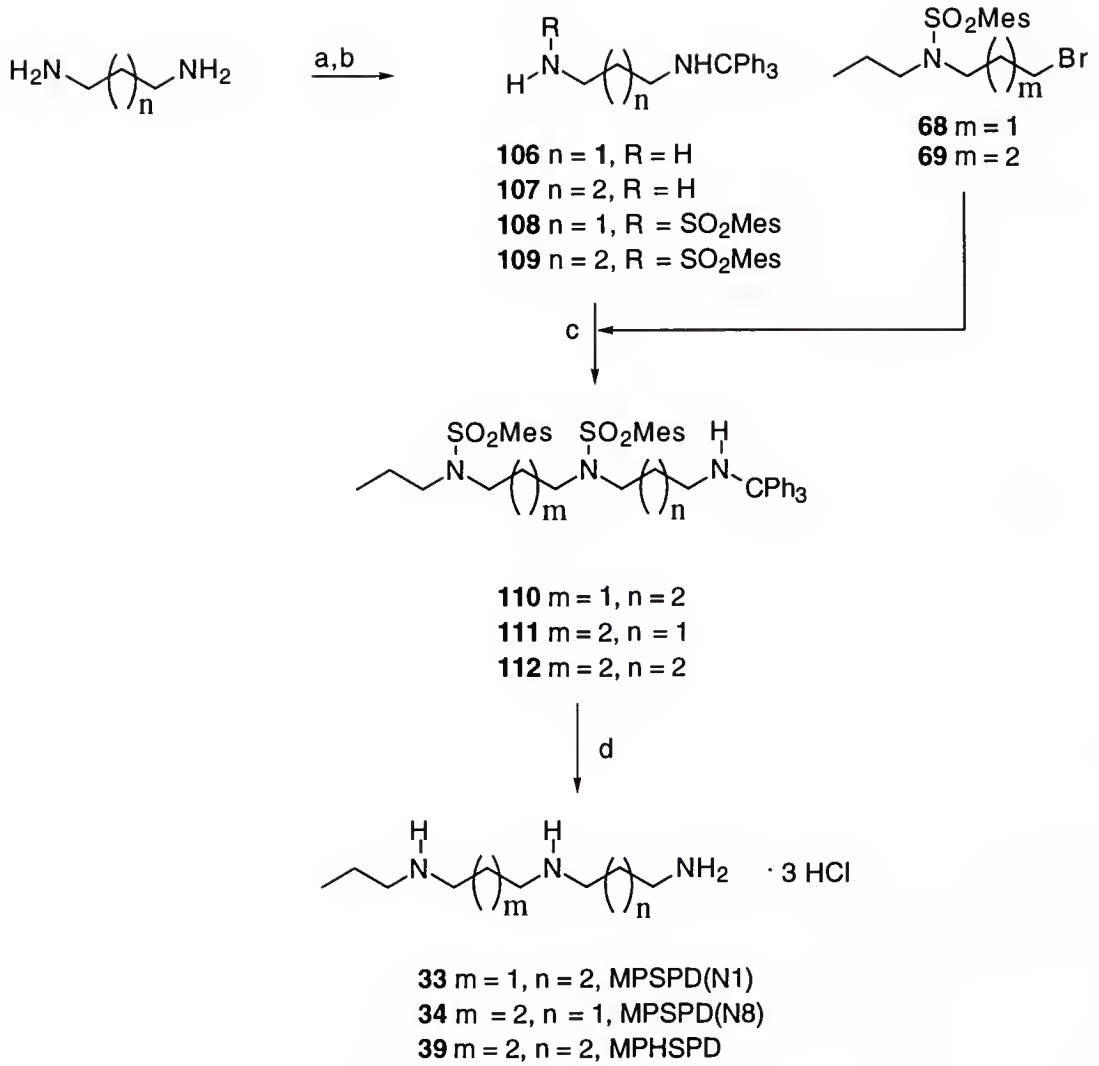
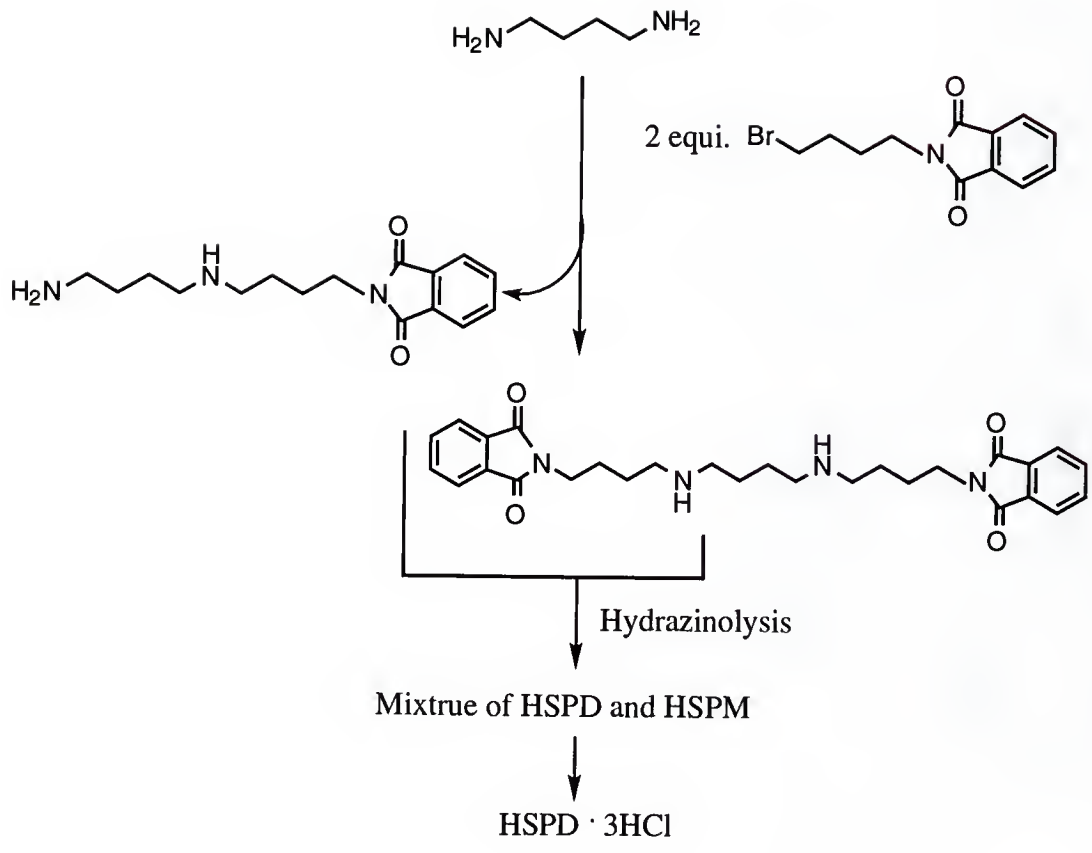
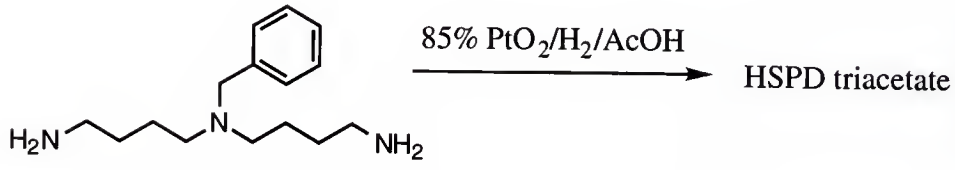


Figure 3-11: Synthesis of monopropyl SPD and HSPD analogues.
(a) Ph_3CCl/CH_2Cl_2; (b) Mesitylenesulfonyl chloride/1 N NaOH(aq)/ CH_2Cl_2; (c) NaH/DMF/**68** or **69**; (d) 30% HBr in HOAc/PhOH/CH_2Cl_2, then HCl.



(A)



(Obtained as in Figure 3-3)

(B)

Figure 3-12: Synthesis of homospermidine by previous methods. (A) Okada's synthesis of HSPD. (B) Bergeron's synthesis of HSPD.

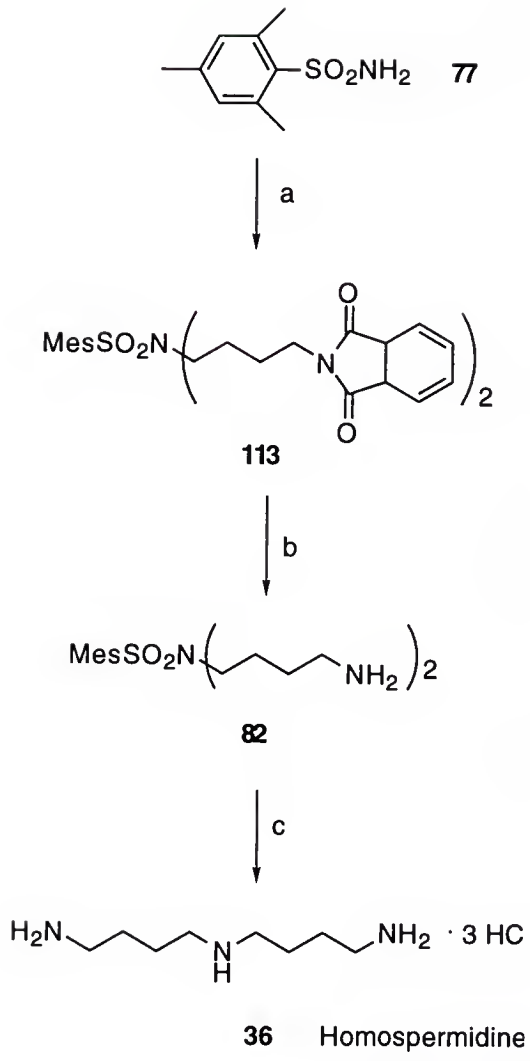


Figure 3-13: Synthesis of homospermidine in this study.
(a) N-(4-Bromobutyl)-phthalimide/NaH/DMF; (b) $(H_2N)_2$, H_2O/EtOH;
(c) 30% HBr in HOAc/PhOH/CH_2Cl_2, then HCl.

Table 3-1: Polyamine analogues synthesized in this study.

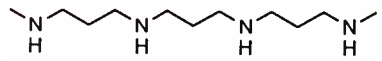
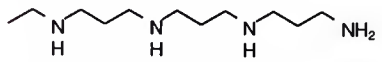
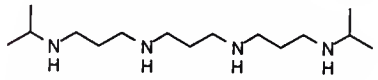
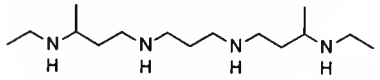
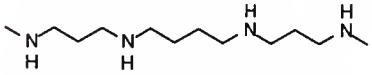
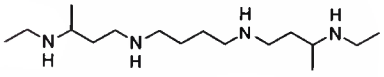
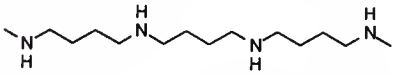
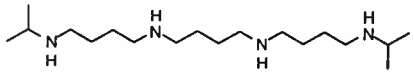
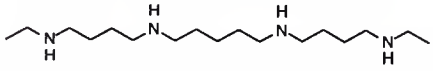
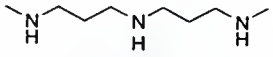
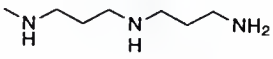
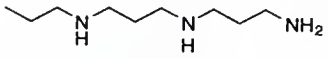
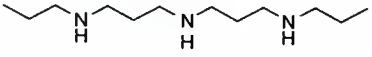
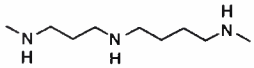
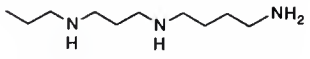
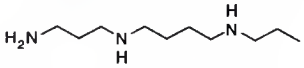
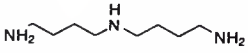
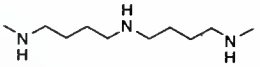
Number	Structure	Abbreviation
Tetraamines		
1		DMNSPM
2		MENSPM
5		DIPNSPM
6		DECroNSPM
7		DMSPM
11		DECroSPM
12		DMHSPM
17		DIPHSPM
20		DE(4,5,4)
Triamines		
23		DMNSPD
24		MENSPD
26		MPNSPD
27		DPNSPD
29		DMSPD
33		MPSPD(N1)
34		MPSPD(N8)
36		HSPD
37		DMHSPD

Table 3-1--continuted

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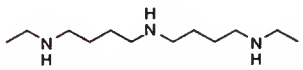
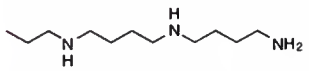
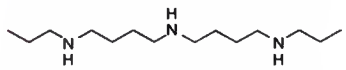
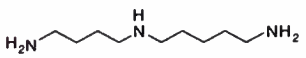
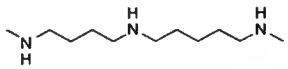
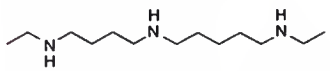
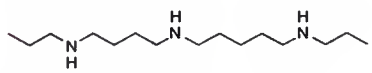
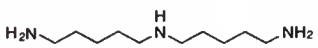
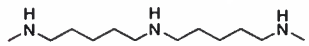
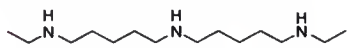
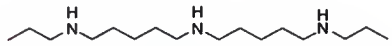
38		DEHSPD
39		MPHSPD
40		DPHSPD
41		4,5-Triamine
42		DM(4,5)
43		DE(4,5)
44		DP(4,5)
45		5,5-Triamine
46		DM(5,5)
47		DE(5,5)
48		DP(5,5)

The numbers of the compounds are as same as the ones assigned in the synthetic methods and in the assays of biological activity.

CHAPTER 4
MATERIALS AND METHODS

Synthetic Methods

Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Reactions using hydride reagents were run in distilled DMF under a nitrogen atmosphere. Fisher optima grade solvents were routinely used, and organic extracts were dried with sodium sulfate. Silica gel 60 (70-230 mesh) obtained from EM Science (Darmstadt, Germany) or silica gel 32-63 (40 mM "flash") from Selecto, Inc. (Kennesaw, GA) was used for column chromatography. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Proton NMR spectra were run at 90 or 300 MHz in $CDCl_3$ (not indicated) or D_2O with chemical shifts given in parts per million downfield from tetramethylsilane or 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt, respectively. Coupling constants (*J*) are in hertz. FAB mass spectra were run in a glycerol/trifluoroacetic acid matrix. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA.

Synthesis of Tetraamine Analogues

***N*-Isopropylmesitylenesulfonamide (63).** A solution of mesitylenesulfonyl chloride (36.5 g, 0.167 mol) in CH_2Cl_2 (200 mL) was added dropwise to isopropylamine (9.87 g, 0.167 mol) in 1 N NaOH (200 mL) at 0 °C. After the mixture was stirred at room temperature overnight, the bilayer was separated and the aqueous layer was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic fraction was then washed with H_2O (100 mL), saturated NaCl solution (100 mL) and dried over anhydrous Na_2SO_4 for 12 h. After the solvent was removed, the crude product was recrystallized from aqueous EtOH to give 33.5 g (83%) of **63** as colorless prisms: mp 97-98 °C; ^{1}H NMR δ 1.00 (d, 6 H, *J* =

7), 2.20 (s, 3 H), 2.55 (s, 6 H), 3.16-3.55 (m, 1 H), 4.35 (d, 1 H, J = 7), 6.85 (s, 2 H). Anal. Calcd. for $C_{12}H_{19}NO_2S$: C, 59.72; H, 7.94; N, 5.80. Found: C, 59.66; H, 7.97; N, 5.81.

***N*-(4-Bromobutyl)-*N*-methylmesitylenesulfonamide (65).** NaH (80%, 3.73 g, 0.124 mol) was added to a solution of **61** (Schreinemakers 1897) (20.4 g, 95.7 mmol) in DMF (200 mL) at 0 °C. The mixture was stirred at room temperature for 30 min, and 1,4-dibromobutane (137 mL, 1.15 mol) was added at 0 °C. After stirred at 70 °C overnight, the reaction was quenched by adding dist. water (10 mL). The solvents were removed under high vacuum and the obtained mixture was taken up in $CHCl_3$ (80 mL) and H_2O (80 mL), followed by extraction of aqueous phase with chloroform (3 x 60 mL). After washed with brine (100 mL), the combined organic portion was then dried over anhydrous sodium carbonate. The solvent was removed by rotvap. Purification by column chromatography with 7:1 hexane/EtOAc produced 9.55 g (30%) of **65** as an oil: ^{1}H NMR δ 1.54-1.85 (m, 4 H), 2.25 (s, 3 H), 2.56 (s, 6 H), 2.68 (s, 3 H), 3.00-3.35 (m, 4 H), 6.88 (s, 2 H). Anal. Calcd. for $C_{14}H_{22}BrNO_2S$: C, 48.28; H, 6.37; N, 4.02. Found: C, 48.50; H, 6.45; N, 3.99.

***N*-(4-Bromobutyl)-*N*-ethylmesitylenesulfonamide (66).** Sodium hydride (80%, 3.71 g, 0.124 mol), **62** (Schreinemakers 1897) (21.6 g, 95.0 mmol) in DMF (100 mL), and 1,4-dibromobutane (140 mL, 1.17 mol) in DMF were combined, and the reaction was worked up by the method of **65**. Column chromatography (6:1 hexane/EtOAc) resulted in 33.2 g (96%) of **66** as an oil: ^{1}H NMR δ 1.04 (t, 3 H, J = 7), 1.4-1.8 (m, 4 H), 2.25 (s, 3 H), 2.55 (s, 6 H), 3.03-3.38 (m, 6 H), 6.87 (s, 2 H). Anal. Calcd. for $C_{15}H_{24}BrNO_2S$: C, 49.73; H, 6.68; N, 3.87. Found: C, 49.78; H, 6.72; N, 3.88.

***N*-(3-Chloropropyl)-*N*-isopropylmesitylenesulfonamide (70).** NaH (80%, 1.7 g, 57 mmol), **63** (9.46 g, 32.9 mmol) in DMF (120 mL), and 1,3-dichloropropane (38 mL, were reacted and worked up following the method of **65.** Column chromatography (12:1 hexane/EtOAc) produced 7.48 g (60%) of **70** as an oil: ^{1}H NMR δ 1.10 (d, 6 H, J = 7),

1.72-2.02 (m, 2 H), 2.22 (s, 3 H), 2.52 (s, 1 H), 3.80 (septet, 1 H, J = 7), 6.80 (s, 2 H). Anal. Calcd. for $C_{15}H_{24}ClNO_2S$: C, 56.68; H, 7.61; N, 4.41. Found: C, 56.74; H, 7.59; N, 4.46.

***N*-(4-Bromobutyl)-*N*-isopropylmesitylenesulfonamide (71).** NaH (80%, 1.54 g, 51.2 mmol), **63** (9.5 g, 39 mmol) in DMF (120 mL), and 1,4-dibromobutane (56.5 mL, 0.473 mol) were mixtured and worked up following the procedure of **65**. Column chromatography (10:1 hexane/EtOAc) furnished 11.34 g (77%) of **71** as an oil: ^{1}H NMR δ 1.16 (d, 6 H, J = 7), 1.50-1.85 (m, 4 H), 2.27 (s, 3 H), 2.50 (s, 6 H), 3.00-3.40 (m, 4 H), 3.75-4.05 (septet, 1 H), 6.90 (s, 2 H). Anal. Calcd. for $C_{16}H_{26}BrNO_2S$: C, 51.06; N, 6.96; N, 3.72. Found: C, 51.17; H, 7.01; N, 3.66.

***N*^{1},*N*^{4},*N*^{8},*N*^{11}-Tetrakis(mesitylenesulfonyl)norspermine (49).** Mesitylenesulfonyl chloride (24.2 g, 0.110 mol) and *N, N'*-bis(3-aminopropyl)-1,3-propanediamine (NSPM, 5.2 g, 28 mmol) in CH_2Cl_2 (150 mL) and 1 N NaOH (150 mL) were combined and worked up by the method of **63**. Recrystallization from 50% EtOAc/hexane afforded 20.1 g (80%) of **49** as a crystalline solid: mp 135-136 °C; ^{1}H NMR δ 1.45-1.80 (m, 6 H), 2.25 (s, 12 H), 2.50 and 2.55 (2 s, 24 H), 2.63-3.27 (m, 12 H), 4.97 (t, 2 H, J = 7), 6.90 (s, 8 H). Anal. Calcd. for $C_{45}H_{64}N_4O_8S_4$: C, 58.93; H, 7.03; N, 6.11. Found: C, 58.84; H, 7.05; N, 6.16.

***N*^{1},*N*^{4},*N*^{8},*N*^{11}-Tetrakis(mesitylenesulfonyl)-*N*^{1},*N* ^{11}-dimethylnorspermine (54).** Sodium hydride (80%, 2.5 g, 0.083 mol), **49** (24.3 g, 0.026 mol) in DMF (200 mL) and iodomethane (8.1 g, 57 mmol) were combined and worked up by the method of **65**. Flash chromatography (5:1 hexane/EtOAc) produced 15.0 g (60%) of **54** as a white solid: ^{1}H NMR δ 1.55-1.81 (m, 6 H), 2.25 (s, 12 H), 2.50 and 2.56 (2 s, 30 H), 2.85-3.15 (m, 12 H), 6.87 (s, 8 H). Anal. Calcd. for $C_{47}H_{68}N_4O_8S_4$: C, 59.72;H, 7.25; N, 5.93. Found: C, 59.78; H, 7.26; N, 5.90.

***N*^{1},*N*^{11}-Dimethylnorspermine Tetrahydrochloride (1).** HBr (30%) in HOAc (300 mL) was added slowly to a solution of **54** (14.8 g, 15.7 mmol) and phenol (58.9 g,

0.626 mol) in CH_2Cl_2 (150 mL) at 0 °C. After the reaction mixture was stirred for 1 day at room temperature, H_2O (200 mL) was added followed by extraction with CH_2Cl_2 (3 x 200 mL). The aqueous portion was evaporated under high vacuum, and the residue was taken up in 1 N NaOH (10 mL) and 19 N NaOH (10 mL) followed by extraction with $CHCl_3$ (14 x 50 mL). After removal of $CHCl_3$, the residue was taken up in EtOH (100 mL) and acidified with concentrated HCl (10 mL). After the solvents were removed, the solid was recrystallized from aqueous EtOH to give 4.84 g (85%) of **1** as crystals: ^{1}H NMR (D_2O) δ 1.80-2.35 (m, 6 H), 2.70 (s, 6 H), 3.05-3.30 (m, 12 H). Anal. Calcd. for $C_{11}H_{32}Cl_4N_4$: C, 36.48; H, 8.90; N, 15.47. Found: C, 36.20; H, 8.84; N, 15.19.

***N*^{1},*N*^{4},*N*^{8},*N*^{11}-Tetrakis(mesitylenesulfonyl)-*N*^{1}-ethyl norspermine (55).** Sodium hydride (80%, 0.20 g, 6.5 mmol), **49** (6.0 g, 6.5 mmol) in DMF (50 mL) and iodoethane (0.52 mL, 6.5 mmol) were combined and worked up by the method of **65**. Flash chromatography (5:3:2 hexane/CH_2Cl_2/EtOAc) produced 2.17 g (30%) of **55** as a white foam: ^{1}H NMR δ 0.85 (t, 3 H, J = 7), 1.50-1.70 (m, 6 H), 2.23 (s, 12 H), 2.47-2.50 (2 s, 24 H), 2.80-3.20 (m, 14 H), 4.80-5.00 (br s, 1 H), 6.85 (s, 8 H). Anal. Calcd. for $C_{47}H_{68}N_4O_8S_4 \cdot H_2O$: C, 58.60; H, 7.32; N, 5.82. Found: C, 58.61; H, 7.25; N, 5.57.

***N*^{1}-Ethylnorspermine Tetrahydrochloride (2).** HBr (30%) in HOAc (50 mL), **55** (0.86 g, 0.91 mmol) and phenol (4.7 g, 50 mmol) in CH_2Cl_2 (100 mL) were mixed at 0 °C. The reaction mixture was stirred overnight at 74 °C and worked up following the procedure of **1**. Recrystallization from aqueous EtOH generated 60.5 mg of **2** (18%) as crystals: ^{1}H NMR (D_2O) δ 1.25 (t, 3 H, J = 7), 1.90-2.30 (m, 6 H), 2.95-3.30 (m, 14 H). Anal. Calcd. for $C_{11}H_{32}Cl_4N_4$: C, 36.48; H, 8.90; N, 15.47. Found: C, 36.55; H, 8.90; N, 15.39.

***N*,*N*'-Bis(mesitylenesulfonyl)-1,3-propanediamine (51).** Mesitylenesulfonyl chloride (28.4 g, 0.130 mol) in CH_2Cl_2 (150 mL) and 1,3-diaminopropane (4.84 g, 65 mmol) in 0.5 N NaOH (300 mL) were combined and the biphasic mixture was stirred at room temperature overnight. The obtained white solid was filtered and washed with 1 N

HCl (3 x 25 mL) and H_2O (3 x 50 mL). Recrystallization from EtOAc furnished 26.1 g (91%) of **51** as crystals: mp 214-215 °C; ^{1}H NMR δ 2.20 (s, 6 H), 2.45 (s, 12 H), 2.60 (quartet, 4 H), 3.25 (s, 2 H), 6.93 (s, 4 H). Anal. Calcd. for $C_{21}H_{30}N_2O_4S_2$: C, 57.51; H, 6.89; N, 6.39. Found: C, 57.43; H, 6.92; N, 6.30.

***N*^{1},*N*^{4},*N*^{8},*N*^{11}-Tetrakis(mesitylenesulfonyl)-*N*^{1},*N*^{11}-diisopropylnorspermine (56).** Sodium hydride (80%, 0.74 g, 23.6 mmol), **51** (3.11 g, 7.14 mmol) in DMF (40 mL) and **70** (5 g, 15.7 mmol) in DMF (40 mL) were combined and worked up following the procedure of **65**. Column chromatography (12:1 toluene/EtOAc) produced 5.3 g (74%) of **56** as an oil: ^{1}H NMR δ 1.05 (d, 12 H, J = 7), 1.50-1.70 (m, 6 H), 2.25 (s, 12 H), 2.50 (s, 24 H), 2.80-3.05 (m, 12 H), 3.75 (septet, 2 H, J = 7), 6.90 (s, 8 H). Anal. Calcd. for $C_{51}H_{76}N_4O_8S_4$: C, 61.17; H, 7.65; N, 5.60. Found: C, 61.26; H, 7.67; N, 5.54.

***N*^{1},*N*^{11}-Diisopropylnorspermine Tetrahydrochloride (5).** A solution of **56** (5.2 g, 5.19 mmol) and phenol (18.4 g, 0.196 mol) in CH_2Cl_2 (80 mL) was combined with 30% HBr in HOAc (100 mL) and worked up following the procedure of **1.** Recrystallization from aqueous EtOH gave 1.5 g (69%) of **5** as crystals: ^{1}H NMR (D_2O) δ 1.30 (t, 12 H, J = 7), 1.90-2.30 (m, 6 H), 3.05-3.60 (m, 14 H). Anal. calcd. for $C_{15}H_{40}Cl_4N_4$: C, 43.07; H, 9.64; N, 13.39. Found: C, 42.92; H, 9.65; N, 13.25.

***N*,*N*'-Bis(mesitylenesulfonyl)-1,4-butanediamine (52).** Mesitylenesulfonyl chloride (54.4 g, 0.249 mol) in CH_2Cl_2 (300 mL) was added to 1,4-diaminobutane (11.34 g, 0.129 mol) in 1 N NaOH (300 mL), and the mixture was stirred for 1 day at room temperature. Organic solvent was evaporated, and 2.4 N HCl (250 mL) was added. Solid was filtered, washed with water (250 mL), and recrystallized from aqueous EtOH to give 50.46 g (90%) of **52** as needles: mp 156.5-157.5 °C; ^{1}H NMR δ 1.36-1.60 (m, 4 H), 2.27 (s, 6 H), 2.57 (s, 12 H), 2.69-2.96 (m, 4 H), 4.65 (t, 2 H, J = 6), 6.89 (s, 4 H). Anal. calcd. for $C_{22}H_{32}N_2O_4S_2$: C, 58.38; H, 7.13; N, 6.19. Found: C, 58.31; H, 7.19; N, 6.14.

***N*^{1},*N*^{5},*N*^{10},*N*^{14}-Tetrakis(mesitylenesulfonyl)-*N*^{1},*N*^{14}-dimethylhomospermine (58).** Sodium hydride (80%, 1.19 g, 39.7 mmol), **52** (5.60 g, 12.4 mmol), and **65** (9.55 g,

27.4 mmol) in DMF (100 mL) were combined and worked up following the procedure of **65**. Purification by flash chromatography (5:1 toluene/EtOAc) gave 8.61 g (70%) of **58** as a white solid: ^{1}H NMR δ 1.25-1.50 (m, 12 H), 2.25 (s, 12 H), 2.53 and 2.59 (2 s, 30 H), 2.90-3.15 (m, 12 H), 6.90 (s, 8 H). Anal. Calcd. for $C_{50}H_{74}N_4O_8S_4$: C, 60.82; H, 7.55; N, 5.67. Found: C, 60.92; H, 7.56; N, 5.66.

***N*^{1},*N*^{14}-Dimethylhomospermine Tetrahydrochloride (12).** A solution of **58** (8.58 g, 8.61 mmol) and phenol (32.6 g, 0.347 mol) in CH_2Cl_2 (70 mL) was mixed with HBr (30%) in HOAc (170 mL) and worked up by the method of **1**. Recrystallization from aqueous EtOH generated 2.54 g (72%) of **12** as white crystalline plates: ^{1}H NMR (D_2O) δ 1.62-1.90 (m, 12 H), 2.68 (s, 6 H), 2.95-3.20 (m, 12 H). Anal. Calcd. for $C_{14}H_{38}Cl_4N_4$: C, 41.59; H, 9.47; N, 13.86. Found: C, 41.66; H, 9.51; N, 13.81.

***N*^{1},*N*^{5},*N*^{10},*N*^{14}-Tetrakis(mesitylenesulfonyl)-*N*^{1},*N*^{14}-diisopropyl-homospermine (59).** Sodium hydride (80%, 1.21 g, 40.3 mmol), **52** (5.70 g, 12.6 mmol) in DMF (50 mL), and **71** (11.34 g, 30.1 mmol) in DMF (75 mL) werecombined and worked up by the method of **65**. Column chromatography (10:1 toluene/EtOAc) gave 7.61 g (58%) of **59** as a white solid: ^{1}H NMR δ 1.10-1.45 (m, 24 H), 2.25 and 2.35 (2 s, 12 H), 2.55 (s, 24 H), 3.65-3.95 (m, 2 H), 6.85 (s, 8 H). Anal. Calcd. for $C_{54}H_{82}N_4O_8S_4$: C, 62.16; H, 7.92; N, 5.37. Found: C, 62.38; H, 7.97; N, 5.22.

***N*^{1},*N*^{14}-Diisopropylhomospermine Tetrahydrochloride (15).** A solution of **59** (7.57 g, 7.25 mmol) and phenol (27.2 g, 0.289 mol) in CH_2Cl_2 (100 mL) was combined with 30% HBr/HOAc (140 mL) and worked up by the method of **1**, recrystallization from aqueous EtOH gave 2.52 g (75%) of **15** as crystals: ^{1}H NMR (D_2O) δ 1.25 (d, 12 H, J = 7), 1.70-1.90 (m, 12 H), 2.95-3.15 (m, 12 H), 3.40 (septet, 2 H, J = 7). Anal. Calcd. for $C_{18}H_{46}Cl_4N_4$: C, 46.96; H, 10.07; N, 12.17. Found: C, 46.69; H, 10.14; N, 12.10.

***N,N'*-Bis(mesitylenesulfonyl)-1,5-pentanediamine (53).** Mesitylenesulfonyl chloride (12.6 g, 57.6 mmol) in CH_2Cl_2 (150 mL) and 1,5-diaminopentane dihydrochloride (5.05 g, 28.8 mmol) in 1 N NaOH (150 mL) were combined and worked up by the method of **52**. Recrystallization from aqueous EtOH gave 12.21 g (91%) of **53** as crystals: mp 117-120 °C; ^{1}H NMR δ 1.18-1.49 (m, 6 H), 2.25 (s, 6 H), 2.58-2.97 (m + s, 16 H), 4.66 (t, 2 H, J = 6), 6.87 (s, 4 H). Anal. Calcd. for $C_{23}H_{34}N_2O_4S_2$: C, 59.20; H, 7.34; N, 6.00. Found: C, 59.13; H, 7.35; N, 6.00.

3,8,14,19-Tetrakis(mesitylenesulfonyl)-3,8,14,19-tetraazaheneicosane (60). NaH (80%, 0.35 g, 12 mmol), **53** (2.29 g, 4.90 mmol) and NaI (63 mg, 0.42 mmol) in DMF (90 mL) was combined with **66** (Bergeron et al. 1994) (5.90 g, 16.3 mmol) in DMF (10 mL) and worked up by the method of **65**. Column chromatography (1.5% $CH_3OH/CHCl_3$) gave 4.40 g (87%) of **60**: ^{1}H NMR δ 0.97 (t, 6 H, J = 7), 1.2-1.5 (m, 14 H), 2.25 (s, 12 H), 2.52 (s, 24 H), 2.8-3.2 (m, 16 H), 6.87 (s, 8 H). Anal. Calcd. for $C_{53}H_{80}N_4O_8S_4$: C, 61.84; H, 7.83; N, 5.44. Found: C, 61.55; H, 7.81; N, 5.30.

3,8,14,19-Tetraazaheneicosane Tetrahydrochloride (20). A solution of **60** (4.29 g, 4.17 mmol) and phenol (14.8 g, 0.157 mol) in CH_2Cl_2 (60 mL) and 30% HBr/HOAc (80 mL) were combined and worked up by the method of **1**. Recrystallization from aqueous EtOH gave 1.31 g (70%) of **20** as plates: ^{1}H NMR (D_2O) δ 1.25 (t, 6 H, J = 7), 1.55-1.85 (m, 14 H), 2.90-3.20 (m, 16 H). Anal. Calcd. for $C_{17}H_{44}Cl_4N_4$: C, 45.74; H, 9.94; N, 12.55. Found: C, 45.80; H, 10.00; N, 12.46.

3-Methyl-4-azahexyl nitrile (72). To a 0 °C mixture of ethylamine hydrochloride (50 g, 0.613 mol) and NaOH (aqueous 50%w/w, 44 mL, 0.613 mol) solution, crotononitrile(cis & trans) (28.5 mL, 0.35 mol) was added dropwise. After stirred at room temperature for 1 h and 60 °C overnight, the reaction was worked up by adding ethyl ether (100 mL) and 1 N NaOH (50 mL) to the mixture to make the aqueous layer pH >13. Then the water layer was separated and extracted with Et_2O (3 x 100 mL).

The organic fractions were combined, dried with anhydrous Na_2SO_4 and purified by short path distillation. 25.40 g (64.6%) **72** was obtained as clear liquid.

4-Methyl-1,5-diazaheptane Dihydrochlodide (73). W-2 grade Raney nickel (18.0 g) and concentrated NH_4OH (60 mL) were successively added to a solution of **72** (25.40 g, 0.226 mol) in CH_3OH (200 mL) in a 500 mL Paee bottle, and a slow stream of NH_3 was bubbled through the mixture for 50 min at 0 °C. After hydrogenation was carried out at 50-60 psi for 18 h, the suspension was filtered through Celite and the solvents were removed in vacuo. The residue was taken up in abs. ethanol and acidified to pH =1 with conc. HCl. 39.25 g (92%) **73** was generated as very hydroscopic white solid: ^{1}H NMR (D_2O) δ 1.28-1.38 (m, 6 H), 1.88-2.01 (m, 1 H), 2.14-2.25 (m, 1 H), 3.10-3.18 (m, 4 H), 3.40-3.46 (m, 1 H). Anal. Calcd. for $C_6H_{18}Cl_2N_2$: C, 38.10; H, 9.59; N, 14.81. Found: C, 38.14; H, 9.63; N, 14.78.

4-Methyl-1,5-bis(mesitylenesulfonyl)-1,5-diazaheptane (74). To a solution of **73** (5 g, 26.4 mmol) in 1 N NaOH (120 mL), 11.6 g (0.0528 mol) 2-mesitylenesulfonyl chloride in CH_2Cl_2 (100 mL) was added dropwise at 0 °C in 45 min. After stirred at room temperature overnight, worked out following the procedure of **63** and purified by recrystallization from EtOAc. 10.26 g (81%) **74** was obtained as shinning plates: m.p 134-136 °C. ^{1}NMR δ 0.90-1.10 (m, 6 H), 1.45-1.70 (m, 2H), 2.20 (s, 6 H), 2.50-2.55 (2s, 12 H), 2.80-3.30 (m, 4 H), 3.70-3.90 (m, 1 H), 5.05 (br t, 1 H), 6.85 (s, 4 H). Anal. Calcd. for $C_{24}H_{36}N_2O_4S_2$: C, 59.97; H, 7.55; N, 5.83. Found: C, 59.93; H, 7.57; N, 5.86.

4,14-Dimethyl-3,7,11,15-tetrakis(mesitylenesulfonyl)-3,7,11,15-tetraazaheptadecane (75). Sodium hydride (80% in oil, 0.9 g, 30 mmol), **74** (10.0 g, 21 mmol) in dry DMF (100 mL).and 1,3-dibromopropane (1 mL, 9.85 mmol) in DMF (10 mL) were combined and worked up by the method of **65**. Column chromatography (3:1 Hexane/EtOAc) produced 5.5 g (56%) **75** as a white foam. ^{1}H NMR δ 0.85-1.05 (m, 12 H), 1.45-1.70 (m, 6H), 2.25 (s, 12 H), 2.50-2.55 (2 s, 18 H), 2.85-3.20 (m, 12 H), 3.35-

3.60 (m, 2 H), 6.85 (s, 8 H). Anal. Calcd. for $C_{51}H_{76}N_4O_8S_4$: C, 61.17; H, 7.65; N, 5.60. Found: C, 61.27; H, 7.65; N, 5.54.

4,14-Dimethyl-3,7,11,15-tetraazaheptadecane Tetrahydrochloride (DECroNSPM, 6). HBr (30%) in HOAc (100 mL), **75** (5.50 g, 5.5 mmol) and phenol (19.8 g, 0.21 mol) in CH_2Cl_2 (90 mL) were combined and worked up following the procedure of **1**. Recrystallized from aqueous EtOH to give 1.59 g (70%) of **6** as crystals: ^{1}H NMR (D_2O) δ 1.15-1.35 (m, 12 H), 1.60-2.30 (m, 6 H), 2.95-3.50 (m, 14 H). Anal. Calcd. for $C_{15}H_{40}N_4Cl_4$: C, 43.07; H, 9.64; N, 13.39. Found: C, 42.97; H, 9.65; N, 13.32.

4,15-Dimethyl-3,7,12,16-tetrakis(mesitylenesulfonyl)-3,7,12,16-tetraazaoctadecane (76). Sodium hydride (60% in oil, 0.48 g, 12 mmol), **74** (4.81 g, 10 mmol) in dry DMF (80 mL), and 1,4-dibromobutane (1.06 g, 4.92 mmol) in DMF (10 mL) were combined and worked up by the method of **65**. Column chromatography (3:1 Toluene/EtOAc) produced 4.0 g (80%) **76** as a white foam: ^{1}H NMR δ 0.85-1.70 (m, 20 H), 2.25 (s, 12 H), 2.50 (s, 18 H), 2.85-3.60 (m, 14 H), 6.85 (s, 8 H). Anal. Calcd. for $C_{52}H_{78}N_4O_8S_4$: C, 61.51; H, 7.74; N, 5.52. Found: C, 61.35; H, 7.70; N, 5.44.

4,15-Dimethyl-3,7,12,16-tetraazaoctadecane Tetrahydrochloride (DECroSPM, 11). HBr (30%) in HOAc (75 mL), **76** (3.98 g, 3.92 mmol) and phenol (13.9 g, 0.15 mol) in CH_2Cl_2 (75 mL) were worked up following the procedure of **1**. Recrystallized from aqueous EtOH to give 1.23 g (73%) of **11** as crystals: ^{1}H NMR (D_2O) δ 1.15-1.45 (m, 12 H), 1.65-2.30 (m, 8 H), 2.95-3.50 (m, 14 H). Anal. Calcd. for $C_{16}H_{42}N_4Cl_4$: C, 44.45; H, 9.79; N, 12.96. Found: C, 44.37; H, 9.72; N, 12.98.

Synthesis of Triamines

***N*^{1},*N*^{4},*N*^{7}-Tris(mesitylenesulfonyl)norspermidine (85).** A solution of norspermidine (2.62 g, 0.02 mol) in 1 N NaOH (80 mL) and 14.0 g (0.064 mol) 2-mesitylenesulfonyl chloride in CH_2Cl_2 (50 mL) were combined and worked up by the

method of **63**. Recrystallization from EtOAc-hexane gave **85** (11.63 g, 86%) as crystalline solid : ^{1}H NMR δ 1.55 (t, 4 H, J = 7), 2.25 (s, 9 H), 2.50-2.55 (2 s, 18 H), 2.70-3.20 (m, 8 H), 4.20 (br t, 2 H), 6.87 (s, 1 H). Anal. Calcd for $C_{33}H_{47}N_3O_6S_3$: C, 58.47; H, 6.99; N, 6.20. Found: C, 58.36; H, 6.95; N, 6.18.

***N*^{1},*N*^{4},*N*^{7}-Tris(mesitylenesulfonyl)-*N*^{1},*N*^{7}-dimethylnorspermidine (90).** NaH (60% in oil, 0.44 g, 11 mmol), **85** (3.39 g, 5 mmol) in DMF (70 mL), and iodomethane (1.63 g, 11.5 mmol) were combined and worked up using the method of **65,** and purified by flash column chromatography with toluene/EtOAc (8:1) as eluant. 2.47 g (70%) **90** was obtained as a thick oil: ^{1}H NMR δ 1.73 (quintet, 4 H), 2.29 (s, 6 H), 2.54-2.55 (2 s, 18 H), 2.60 (s, 6 H), 2.99-3.06 (2 t, 8 H, J = 7), 6.94 (s, 6 H). Anal. Calcd for $C_{35}H_{51}N_3O_6S_3$: C, 59.55; H, 7.28; N, 5.95. Found: C, 59.50; H, 7.33; N, 5.88.

***N*^{1},*N*^{7}-Dimethylnorspermidine Trihydrochloride (23).** A solution of **90** (2.13 g, 3.02 mmol) and phenol (12.27 g) in CH_2Cl_2 and HBr (30% in HOAc, 30 mL) were combined and worked up by the method of **1**. Recrystallization from aqueous EtOH afforded 0.298 g (37%) of **23** as plates: ^{1}H NMR (D_2O) δ 2.08-2.19 (m, 4 H), 2.75 (s, 6 H), 3.13-3.22 (m, 8 H). Anal. Calcd for $C_8H_{24}N_3Cl_3$: C, 35.77; H, 9.00; N, 15.64. Found: C, 35.91; H, 8.96; N, 15.69.

***N*^{1},*N*^{4},*N*^{7}-Tris(mesitylenesulfonyl)-*N*^{1}-monopropylnorspermidine (91).** A solution of **85** (2.57 g, 3.8 mmol) in DMF, NaH (60% in oil, 0.52 g), and 1-iodopropane were combined and worked up with the method of **65**. Flash chromatography (hexane/EtOAc 3:1) generated 1.16 g (23%) of **91** as an oil: ^{1}H NMR δ 0.66 (t, 3 H, J = 7), 1.23-1.31 (m, 2 H), 1.58-1.62 (m, 4 H), 2.262-2.271 (2 s, 9 H), 2.507-2.519 (2 s, 12 H), 2.59 (s, 6 H), 2.82-2.99 (m, 8 H), 3.21 (t, 2 H, J = 7), 4.85 (br t, 1 H), 6.89-6.92 (m, 6 H). Anal. Calcd for $C_{36}H_{53}N_3O_6S_3$: C, 60.05; H, 7.42; N, 5.84. Found: C, 59.79; H, 7.32; N, 5.70.

***N*^{1}-Monopropylnorspermidine Tihydrochloride (26).** HBr (30% in HOAc, 30 mL), **91** (1.14 g, 1.58 mmol) and phenol (6.4 g) in CH_2Cl_2 were reacted and worked up

following the procedure of **1**. Recrystallization from aqueous EtOH gave **26** (87 mg, 20%) as crystals: ^{1}H NMR (D_2O) δ 0.98 (t, 3 H, J = 7.5), 1.67-1.75 (m, 2 H), 2.07-2.16 (m, 4 H), 3.01- 3.21 (m, 10 H). Anal. Calcd for $C_9H_{26}Cl_3N_3$: C, 38.24; H, 9.27; N, 14.87. Found: C, 38.15; H, 9.32; N, 14.75.

***N*^{1},*N*^{4},*N*^{7}-Tris(mesitylenesulfonyl)-*N*^{1},*N*^{7}-dipropylnorspermidine (92).** NaH (60%, 0.44 g, 11 mmol), **85** (3.39 g, 5 mmol) and 1-iodopropane (1.95 g, 11.5 mmol) in DMF (70 mL) were combined and worked up following the procedure of **65**. Column chromatography (3:1 hexane/EtOAc) gave 3.54 g (93%) of **92** as an thick oil: ^{1}H NMR δ 0.7 (s, 6 H), 1.20-1.65 (m, 8 H), 2.25 (s, 9 H), 2.50 (s, 18 H), 2.80-3.05 (m, 12 H), 6.87 (s, 6 H). Anal. Calcd for $C_{39}H_{59}N_3O_6S_3$: C, 61.55; H, 7.68; N, 5.52. Found: C, 61.52; H, 7.79; N, 5.55.

***N*^{1},*N*^{7}-Dipropylnorspermidine Trihydrochloride (27).** HBr in HOAc (30%, 80 mL), **91** (3.475 g, 4.57 mmol) and phenol (15.8 g) in CH_2Cl_2 (30 mL) were reacted and worked up following the procedure of **1**. Recrystallization from aqueous EtOH gave 1.26 g (85%) of **27** as plates: ^{1}H NMR (D_2O) δ 0.87 (t, 6 H, J = 7), 1.60 (m, 4 H), 2.01 (m, 4 H), 2.93 (t, 4 H, J = 7), 3.06 (m, 8 H). Anal. Calcd for $C_{12}H_{32}Cl_3N_3$: C, 44.38; H, 9.93; N, 12.94. Found: C, 44.42; H, 9.89; N, 12.88.

***N*^{1},*N*^{4},*N*^{8}-Tri(mesitylenesulfonyl)spermidine (86).** 2-Mesitylenesulfonyl chloride (6.87 g, 31.4 mmol) in CH_2Cl_2 (30 mL) was combined with spermidine trihydrochloride (**28**) and worked up with the method of **63**. Flash column chromatography (4:3 hexane/EtOAc) gave 3.73 g (55%) **86** as a white foam: ^{1}H NMR δ 1.30 (m, 2 H), 1.44 (m, 2 H), 1.66 (m, 2 H), 2.30 (s, 9 H), 2.46 (s, 6 H), 2.60 (s, 12 H), 2.76 (quartet, 2 H), 2.84 (quartet, 2 H), 3.04 (t, 2 H, J = 7), 3.24 (t, 2 H, J = 7), 4.56 (br t, 1 H), 4.92 (br t, 1 H), 6.90 (s, 2 H), 6.95 (s, 4 H). Anal. Calcd for $C_{34}H_{49}N_3O_6S_3$: C, 59.02; H, 7.14; N, 6.07. Found: C, 58.74; H, 7.12; N, 5.99.

***N*^{1},*N*^{4},*N*^{8}-Tri(mesitylenesulfonyl)-*N*^{1},*N*^{8}-dimethylspermidine (93).** NaH (60%, 0.41 g, 10.3 mmol), **86** (2.15 g, 3.11 mmol), and iodomethane (1.41 g, 9.95 mmol,

620 mL) in DMF (60 mL) were combined and worked up by the method of **65.** Flash chromatography (5:3 hexane/EtOAC) gave 2.24 g **93** (100%) as a thick oil: ^{1}H NMR δ 1.39-1.43 (m, 4 H), 1.69-1.78 (m, 2 H), 2.28 (s, 3 H), 2.30 (s, 6 H), 2.55 (s, 12 H), 2.57 (s, 6 H), 2.60 (s, 3 H), 2.62 (s, 3 H), 2.96-3.13 (m, 8 H).

***N*^{1},*N*^{8}-Dimethylspermidine Trihydrochloride (29).** HBr (30%) in HOAc (60 mL), **93** (2.24 g, 3.11 mmol) and phenol (12.3 g) in CH_2Cl_2 (30 mL) were combined and worked up by the procedure of **1**. Recrystallization from aqueous EtOH provided 0.658 g (75%) **29** as crystals: ^{1}H NMR (D_2O) δ 1.77-1.82 (m, 4 H), 2.06-2.18 (m, 2 H), 2.73 (s, 3 H), 2.75 (s, 3 H), 3.06-3.19 (m, 8 H). Anal. Calcd. for $C_9H_{26}Cl_3N_3$: C, 38.24; H, 9.27; N, 14.86. Found: C, 38.19; H, 9.28; N, 14.79.

***N*-Bis(3-cyanopropyl)mesitylenesulfonamide (79).** Sodium hydride (60%, 2.6 g, 66 mmol), **77** (6 g, 30 mmol) in DMF (100 mL) and 3-bromobutyronitrile (9.77 g, 66 mmol) were combined and worked up by the method of **65**, column chromatography (1:1 hexane/EtOAc) produced 7.48 g (75%) of **79** as an oil: ^{1}H NMR δ 1.87 (m, 4 H), 2.27 (t, 4 H, J = 7), 2.31 (s, 3 H), 2.60 (s, 6 H), 3.34 (t, 4 H, J = 7), 6.99 (s, 2 H). Anal. Calcd for $C_{17}H_{23}N_3O_2S$: C, 61.24; H, 6.95; N, 12.60. Found: C, 61.08; H, 6.96; N, 12.55.

***N*^{1},*N*^{5},*N*^{9}-Tris(mesitylenesulfonyl)homospermidine (87).** 2-Mesitylene-sulfonyl chloride (6.71 g, 30.7 mmol) in CH_2Cl_2 (30 mL) was reacted with a solution of **82** in 1 N NaOH (35 mL) and worked up by the method of **63**. Column chromatography (4:1 toluene/EtOAc) produced 3.06 g (31%) **87** as a white foam: ^{1}H NMR δ 1.32-1.38 (m, 4 H), 1.44-1.54 (m, 4 H), 2.28-2.29 (2 s, 9 H), 2.54 (s, 6 H), 2.60 (s, 12 H), 2.79 (quartet, 4 H), 3.09 (t, 4 H, J = 7), 4.70-4.80 (br s, 2 H), 6.90 (s, 2 H), 6.92 (s, 4 H). Anal. Calcd. for $C_{35}H_{51}N_3O_6S_3$: C, 59.55; H, 7.28; N, 5.95. Found: C, 59.34; H, 7.29; N, 5.92.

***N*^{1},*N*^{5},*N*^{9}-Tris(mesitylenesulfonyl)-*N*^{1},*N*^{9}-dimethylhomospermidine (94).** Sodium hydride (60%, 0.17 g, 4.17 mmol), **87** (1.28 g, 1.8 mmol) in DMF (50 mL), and iodomethane (0.565 g, 0.25 mL, 4.0 mmol) were combined and worked up by the

method of **65**. Column chromatography (2:1 toluene/EtOAc) produced 1.14 g (86%) of **94** as an oil : ^{1}H NMR δ 1.38-1.44 (m, 8 H), 2.28 (s, 3 H), 2.30 (s, 6 H), 2.57 (s, 18 H), 2.62 (s, 6 H), 3.03-3.14 (m, 8 H), 6.93-6.94 (2 s, 6 H). Anal. Calcd. for $C_{37}H_{55}N_3O_6S_3$: C, 60.54; H, 7.55; N, 5.72. Found: C, 60.26; H, 7.61; N, 5.63.

***N*^{1},*N*^{5},*N*^{9}-Tris(mesitylenesulfonyl)-*N*^{1},*N*^{9}-diethylhomospermidine (95).** Sodium hydride (80%, 0.264 g, 8.8 mmol), **77** (0.796 g, 4 mmol) in DMF (60 mL), and*N*-(4-bromobutyl)-*N*-ethyl mesitylenesulfonamide **66** (3.189 g, 8.8 mmol) in DMF (15 mL) were combined and worked up by the method of **65**. Column chromatography (3:1 hexane/EtOAc) gave **95** 2.82 g (92.5%) as an oil: ^{1}H NMR δ 0.96 (t, 6 H, *J* = 7), 1.20-1.40 (m, 8 H), 2.25 (s, 9 H), 2.55 (s, 18 H), 2.85-3.20 (m, 12 H), 6.90 (s, 6 H). Anal. Calcd. for $C_{39}H_{59}N_3O_6S_3$: C, 61.47; H, 7.80; N, 5.51. Found: C, 61.27; H, 7.89; N, 5.44.

***N*^{1},*N*^{5},*N*^{9}-Tris(mesitylenesulfonyl)-*N*^{1},*N*^{9}-dipropyl homospermidine (96).** Sodium hydride (60%, 0.17 g, 4.17 mmol), **87** (1.28 g, 1.8 mmol) in DMF (50 mL), and 1-iodopropane (0.67 g, 0.39 mL, 4.0 mmol) were combined and worked up by the method of **65**. Column chromatography (4:1 hexane/EtOAc) produced 1.26 g (89%) of **96** as an oil: ^{1}H NMR δ 0.74 (t, 6 H, *J* = 7), 1.26-1.45 (m, 12 H), 2.29 (s, 9 H), 2.55 (s, 18 H), 2.98-3.13 (m, 12 H), 6.87 (s, 6 H). Anal. Calcd. for $C_{41}H_{63}N_3O_6S_3$: C, 62.32; H, 8.04; N, 5.32. Found: C, 62.19; H, 8.00; N, 5.33.

***N*^{1},*N*^{9}-dimethylhomospermidine Trihydrochloride (37).** HBr in HOAc (30%, 30 mL), **94** (1.12 g, 1.52 mmol) and phenol (5.40 g, 57 mmol) in CH_2Cl_2 (25 mL) were combined and worked up following the procedure of **1**. Recrystallization form aqueous EtOH gave 354 mg (79%) **37** as plates: ^{1}H NMR (D_2O) δ 1.78 (m, 8 H), 2.73 (s, 6 H), 3.08-3.12 (m, 8 H). Anal. Calcd. for $C_{10}H_{28}Cl_3N_3$: C, 40.48; H, 9.51; N, 14.16. Found: C, 40.45; H, 9.44; N, 14.09.

***N*^{1},*N*^{9}-diethylhomospermidine Trihydrochloride (38).** HBr in HOAc (30%, 20 mL), **95** (1.87 g, 2.454 mmol) and phenol (4.42 g, 49 mmol) in CH_2Cl_2 (20 mL)

were combined and worked up following the procedure of **1**. Recrystallization form aqueous EtOH gave **38** 493 mg (62%) as plats: ^{1}H NMR (D_2O) δ 1.30 (s, 6 H), 1.55-1.90 (m, 8 H), 2.95-3.20 (m, 12 H). Anal. Calcd. for $C_{12}H_{32}Cl_3N_3$: C, 44.38; H, 9.93; N, 12.94. Found: C, 44.49; H, 9.98; N, 12.96.

***N*^{1},*N*^{9}-dipropylhomospermidine Trihydrochloride (40).** HBr in HOAc (30%, 20 mL), **96** (1.24 g, 1.56 mmol) and phenol (5.40 g, 57 mmol) in CH_2Cl_2 (25 mL)were combined and worked up following the procedure of **1**. Recrystallization form aqueous EtOH gave 430 mg (78%) **40** as plates: ^{1}H NMR (D_2O) δ 0.98 (t, 6 H, *J* = 7.5), 1.70 (m, 4 H), 1.76-1.80 (m, 8 H), 3.02 (t, 4 H, *J* = 7.5), 3.08-3.12 (m, 8 H). Anal. Calcd. for $C_{14}H_{36}Cl_3N_3$: C, 47.66; H, 10.29; N, 11.91. Found: C, 47.70; H, 10.21; N, 11.86.

***N*-(*tert*-Butoxycarbonyl)mesitylenesulfonylamide (102).** Mesitylenesulfonamide **77** (5 g, 25 mmol) and oxalyl chloride (20 g, 0.157 mol, 6.3 equivalent.) were stirred for 24 h at refluxing to give a solid, which was heated at 170 °C for 1 h in o-dichlorobenzene (85 mL). After cooling to room temperature, *tert* -butyl alcohol (2.4 mL, 25 mmol) was added by syringe and stirred at room temperature for 8 h. The mixture was cooled to 0 °C and 1 N NaOH (150 mL) was added. The aqueous phase (pH = 13) was first extracted with $CHCl_3$ (3 x 100 mL), cooled to 0 °C, and then acidified to pH = 2 with cold 1 N HCl (150 mL) . Extraction with $CHCl_3$ (4 x 150 mL), removal of solvent, and purification by flash chromatography (5:1 hexane/EtOAc) gave 4.83 g (64%) of **102** as a white solid: mp 132-135 °C. (lit. 133-135 °C). ^{1}H NMR δ 1.26 (s, 9 H), 2.25 (s, 3 H), 2.60 (s, 6 H), 6.90 (s, 2 H), 7.55 (s, 1 H).

***N*-(5-Bromopentyl)-*N'*-ethylmesitylenesulfonamide (67).** Sodium hydride (80%, 1.26 g, 42 mmol), **62** (6.82 g, 6.82 mmol) in DMF (100 mL), and 1,5-dibromopentane (82.8 g, 49 mL, 0.36 mol) were combined and worked up by the method of **65**. Column chromatography (7:1 hexane/EtOAc) produced 7.87 g (70%) of **67** as an oil: ^{1}H NMR δ 1.00 (t, 3 H, *J* = 7), 1.30-1.75 (m, 6 H), 2.20 (s, 3 H), 2.50 (s, 6

H), 3.02-3.30 (m, 6 H), 6.80 (s, 2 H). High resolution mass spectrum ($C_{16}H_{26}BrNO_2S$): M+H 376.0946; found 376.0960.

N^1,N^4-Bis(mesitylenesulfonyl)-N^1-(tert-butoxycarbonyl)-N^4-ethyl-1,4-butanediamine (103). A solution of **102** (3.45 g, 11.5 mmol) in dry DMF (100 mL), NaH (80%, 0.45 g, 23.4 mmol) and *N*-(4-bromobutyl)-*N'*-ethylmesitylenesulfonamide **66** (5.00 g, 13.8 mmol) (Bergeron et al. 1994) were combined and worked up by the method of **65**. Flash chromatography (20:1 toluene/EtOAc) gave 6.44 g (96%) **103** as an oil: ^{1}H NMR δ 1.12 (t, 3 H, J = 7), 1.20 (s, 9 H), 1.55-1.65 (m, 4 H), 2.29 (s, 2 H), 2.30 (s, 2 H), 2.52 (s, 4 H), 2.62 (s, 4 H), 3.16-3.24 (m, 2 H), 3.30 (quartet, 2 H), 3.70 (m, 2 H), 6.94 (s, 4 H).

N^1,N^4-Bis(mesitylenesulfonyl)-N^4-ethyl-1,4-butanediamine (104). TFA (70 mL) was slowly dripped into the CH_2Cl_2 (30 mL) solution of **103** (6.20 g, 10.6 mmol) at 0 °C. After stirred at 0 °C for 20 min and room temperature for 30 min, the solvent and excess TFA were moved by rotvap and the residue was neutralized to pH > 8 by saturated $NaHCO_3$ solution. After extracted with CH_2Cl_2 (4 x 100 mL), all of the extracts were combined and dried over anhydrous Na_2SO_4 overnight. 5.10 g (100%) of **104** as foam was obtained after removal of the solvent: ^{1}H NMR δ 0.97 (t, 3 H, J = 7), 1.20-1.50 (m, 4 H), 2.25 (s, 6 H), 2.50-2.55 (2 s, 12 H), 2.95-3.25 (m, 6 H), 4.45 (br t, 1 H), 6.85 (s, 4 H). Anal. Calcd. for $C_{24}H_{36}N_2O_4S_2$: C, 59.97; H, 7.55; N, 5.83. Found: C, 59.83; H, 7.56; N, 5.76.

3,8,14-Tris(mesitylenesulfonyl)-3,8,14-Triazahexadecane (105). A solution of **104** (5.10 g, 10.6 mmol) in DMF, sodium hydride (0.41 g, 13.8 mmol), and **67** (4.80 g, 12.7 mmol) in DMF (10 mL) were combined and worked up by the method of **65**. Flash chromatography (12:1 toluene/EtOAc) gave 5.43 g (66%) of **105** as an oil: ^{1}H NMR δ 0.9-1.1 (m, 6 H), 1.2-1.5 (m, 10 H), 2.25 (s, 6 H), 2.30 (s, 3 H), 2.55 (s, 18 H), 2.9-3.2 (m, 12 H), 6.85 (s, 6 H). Anal. Calcd. for $C_{40}H_{61}N_3O_6S_3$: C, 61.90; H, 7.92; N, 5.41. Found: C, 62.03; H, 7.97; N, 5.33.

3,8,14-Triazahexadecane Trihydrochloride (43). HBr in HOAc (30%, 100 mL), **105** and phenol in CH_2Cl_2 (100 mL) were reacted and worked up by the method of **1**. Recrystallization from aqueous EtOH gave 1.63 g (69%) of **43** as plates: ^{1}H NMR (D_2O) δ 1.38 (t, 3 H, *J* = 7), 1.39 (t, 3 H, *J* = 7), 1.60-1.70 (m, 10 H), 3.02-3.15 (m, 12 H). Anal. Calcd. for $C_{13}H_{34}N_3Cl_3$: C, 46.09; H, 10.12; N, 12.40. Found: C, 46.20; H, 10.08; N, 12.47.

***N*-(4-Cyanobutyl)mesitylenesulfonamide (78).** NaH (60%, 2.0 g, 0.05 mmol), **77** (10.0 g, 0.05 mmol) in DMF (100 mL) and 4-bromobutyronitrile (5.92 g, 4 ml, 0.04 mol) were combined and worked up by the method of **65**. Column chromatography (4:3 hexane/EtOAc) produced **78** (5.04 g, 38%) as an oil: ^{1}H NMR δ 1.78 (s, 3 H), 2.25 (s, 3 H), 2.35 (t, 2 H, *J* = 7), 2.95 (quartet, 2 H), 5.05 (br t, 1 H), 6.90 (s, 2 H). Anal. Calcd. for $C_{13}H_{18}N_2O_2S$: C, 58.62; H, 6.81; N, 10.52. Found: C, 58.52; H, 6.86; N, 10.46.

***N*-(4-Cyanobutyl)-*N*-(5-cyanopentyl)mesitylenesulfonamide (80).** Sodium hydride (60%, 0.90 g, 22.6 mmol), a solution of **78** (5.02 g, 18.85 mmol) in DMF and 5-bromovaleronitrile were combined and worked up by the method of **65.** Column chromatography (1:1 hexane/EtOAc) provided 5.20 g (79%) of **80** as an oil: ^{1}H NMR δ 1.49-1.66 (m, 4 H), 1.82 (m, 2 H), 2.22 (t, 2 H, *J* = 7), 2.25 (t, 2 H, *J* = 7), 2.29 (s, 3 H), 2.57 (s, 6 H), 3.19 (t, 2 H, *J* = 7), 3.29 (t, 2 H, *J* = 7), 6.95 (s, 2 H). Anal . Calcd. for $C_{18}H_{25}N_3O_2S$: C, 62.22; H, 7.25; N, 12.09. Found: C, 62.24; H, 7.28; N, 11.99.

6-(Mesitylenesulfonyl)-1,6,12-Triazadodecane (83). W-2 grade Raney nickel (7.60 g) and concentrated NH_4OH (10 mL) were successively added to a solution of **80** (5.06 g, 14.6 mmol) CH_3OH (30 mL) and THF (30 mL) in a 200 mL Parr bottle, and a slow stream of NH_3 was bubbled through the mixture for 30 min at 0 °C. After the hydrogenation was carried out at 50-55 psi for 8 h, the suspention was filtered through Celite and the solvents were removed in vacuo. 4.70 g (91%) of **83** was obtained as an oil: ^{1}H NMR δ 1.14-1.24 (m, 10 H), 2.25 (s, 3 H), 2.6 (s, 6 H), 3.05-3.25 (m, 8 H), 3.45

(s, 4 H), 6.9 (s, 2 H). Anal. Calcd. for $C_{18}H_{33}N_3O_2S \cdot 0.5H_2O$: C, 59.31; H, 9.40; N, 11.53. Found: C, 59.64; H, 9.26; N, 11.19. High resolution mass spectrum ($C_{18}H_{33}N_3O_2S$): M+H 356.2372; found 356.2313.

1,6,12-Triazadodecane Trihydrochloride (41). A solution of **83** (2.43 g 6.83 mmol) and phenol (6 g, 63.8 mmol) in CH_2Cl_2 and 30% HBr in HOAc (33 mL) were combined and worked up by the method of **1**. Recrystallization from aqueous EtOH gave 0.97g (50%) of **41** as a very hydroscopic solid: ^{1}H NMR (D_2O) δ 1.47 (m, 2 H), 1.70-1.80 (m, 8 H), 3.00-3.10 (m, 8 H). Anal. Calcd. for $C_9H_{26}Cl_3N_3$: C, 38.24; H, 9.27; N, 14.87. Found: C, 38.28; H, 9.22; N, 14.82.

1,6,12-Tris(mesitylenesulfonyl)-1,6,12-Triazadodecane (88). Mesitylenesulfonyl chloride (4.29 g, 19.6 mmol) in CH_2Cl_2 (40 mL) and **83** (3.17 g, 8.92 mmol) in 1 N NaOH (20 mL) were reacted and worked up by the method of **63**. Flash chromatography (4:3 hexane/EtOAc) produced 5.66 g (88%) of **88** as an oil: ^{1}H NMR δ 1.12-1.17 (m, 2 H), 1.34-1.51 (m, 8 H), 2.29 (s, 3 H), 2.30 (s, 6 H), 2.55 (s, 6 H), 2.60-2.62 (2 s, 12 H), 2.77-2.81 (m, 4 H), 3.06 (t, 2 H, J = 7), 3.11 (t, 2 H, J = 7), 4.50-4.60 (m, 2 H), 6.92 (s, 2 H), 6.95 (s, 2 H). Anal. Calcd. for $C_{36}H_{53}N_3O_6S_3$: C, 60.05; H, 7.42; N, 5.84. Found: C, 59.88; H, 7.41; N, 5.80. High resolution mass spectrum ($C_{36}H_{53}N_3O_6S_3$): M+H 720.3175; found 720.31932.

2,7,13-Tris(mesitylenesulfonyl)-2,7,13-Triazatetradecane (97). Sodium hydride (60%, 0.28 g, 6.9 mmol), a solution of **88** (2.16 g, 3.0 mmol) in DMF (30 mL) and iodomethane (611 mL, 9.6 mmol) were combined and worked up by the method of **65.** Column chromatography (7:3 hexane/EtOAc) provided 1.90 g (85%) of **97** as an thick oil: ^{1}H NMR δ 1.08-1.16 (m, 2 H), 1.38-1.50 (m, 8 H), 2.28-2.29 (2 s, 9 H), 2.57-2.58 (2 s, 18 H), 2.63 (s, 3 H), 2.65 (s, 3 H), 3.02-3.14 (m, 8 H), 6.95 (s, 6 H). High resolution mass spectrum ($C_{38}H_{57}N_3O_6S_3$): M+H 748.3488; found 748.3483.

2,7,13-Triazatetradecane Trihydrochloride (42). HBr in HOAc (30%, 45 mL), **97** (1.85 g, 2.47 mmol) and phenol (8.5 g) in CH_2Cl_2 (20 mL) were combined and

worked up by the method of **1**. Recrystallization from aqueous EtOH gave 529 mg (69%) of **42** as crystals: ^{1}H NMR (D_2O) δ 1.42-1.52 (m, 2 H), 1.69-1.81 (m, 8 H), 2.73-2.74 (2 s, 6 H), 3.03-3.12 (m, 8 H). Anal. Calcd. for $C_{11}H_{30}Cl_3N_3$: C, 42.52; H, 9.73; N, 13.52. Found: C, 42.52; H, 9.69; N, 13.58.

4,9,15-Tris(mesitylenesulfonyl)-4,9,15-Triazaoctadecane (98). Sodium hydride (60%, 0.273 g, 6.84 mmol), **88** (2.24 g, 3.11 mmol) in DMF (30 mL) and 1-iodopropane (670 mL, 6.84 mmol) were combined and worked up by the method of **65.** Column chromatography (3:1 hexane/EtOAc) provided 2.01 g (80%) of **98** as an thick oil: ^{1}H NMR δ 0.71-0.78 (m, 6 H), 1.01-1.11 (m, 2 H), 1.34-1.48 (m, 12 H), 2.29 (s, 6 H), 2.57-2.58 (2 s, 18 H), 2.98-3.13 (m, 12 H), 6.92 (s, 6 H). Anal. Calcd. for $C_{42}H_{65}N_3O_6S_3 \cdot H_2O$: C, 61.35; H, 8.21; N, 5.11. Found: C, 61.34; H, 8.07; N, 5.05. High resolution mass spectrum ($C_{42}H_{65}N_3O_6S_3$): M+H 804.4114; found 804.4114.

4,9,15-Triazaoctadecane Trihydrochloride (44). HBr in HOAc (30%, 45 mL), **98** (1.99 g, 2.5 mmol) and phenol (8.5 g) in CH_2Cl_2 (20 mL) were combined and worked up by the method of **1**. Recrystallization from aqueous EtOH give 852 mg (83%) of **44** as plate crystals: ^{1}H NMR (D_2O) δ 0.97 (s, 6 H), 1.40-1.51 (m, 2 H), 1.66-1.80 (m, 12 H), 2.98-3.15 (m, 12 H). Anal. Calcd. for $C_{15}H_{38}Cl_3N_3$: C, 49.11; H, 10.44; N, 11.45. Found: C, 49.02; H, 10.40; N, 11.42.

***N*-Bis(4-cyanobutyl)mesitylenesulfonamide (81).** Sodium hydride (80%, 1.22 g, 51 mmol), a solution of **77** (5.0 g, 25 mmol) in DMF (50 mL) and 5-chlorovaleronitrile (6.5 g, 55 mmol) were combined and worked up by the method of **65**. Column chromatography (7:3 hexane/EtOAc) provided 6.31 g (70%) **81** as an oil: ^{1}H NMR δ 1.57 (m, 4 H), 1.66 (m, 4 H), 2.26 (t, 4 H, J = 7), 2.60 (s, 6 H), 3.22 (t, 4 H, J = 7), 6.98 (s, 2 H). Anal. Calcd. for $C_{19}H_{27}N_3O_2S$: C, 63.13; H, 7.53; N, 11.62; S, 8.87. Found: C, 63.31; H, 7.68; N, 11.43; S, 8.97.

7-Mesitylenesulfonyl-1,7,13-triazatridecane (84). W-2 grade Raney nickel (2.4 g) and concentrated NH_4OH (10 mL) were successively added to a solution of **81** (5.69 g,

15.8 mmol) CH_3OH (60 mL) in a 200 mL Parr bottle, and a slow stream of NH_3 was bubbled through the mixture for 30 min at 0 °C. After the hydrogenation was carried out at 50-55 psi for 21 h, more Raney nickel (0.5 g) was added into the mixture, followed with further hydrogenation for 21 h. The suspension was filtered through Celite and the solvents were removed in vacuo. The crude product was purified by a short silicon column using abs. EtOH and 5% NH_4OH/EtOH as eluents, and 5.71 g (98%) of **84** was obtained as a light yellow oil: ^{1}H NMR δ 1.17 (m, 4 H), 1.47 (m, 8 H), 2.27 (s, 3 H), 2.57 (s, 6 H), 2.61 (m, 4 H), 3.13 (t, J = 7.5 Hz, 4 H), 6.91 (s, 2 H). High resolution mass spectrum ($C_{19}H_{35}N_3O_2S$): M+H 370.2528; found 370.2530.

1,7,13-Triazatridecane Trihydrochloride (45). A solution of **84** (2.0 g, 5.42 mmol) and phenol (4.8 g, 51 mmol) in CH_2Cl_2 (40 mL) was combined with 30% HBr in HOAc (26 mL) and the reaction was worked up following the procedure of **1**. Recrystallization from aqueous EtOH gave 0.97 g (61%) **45** as white solid: ^{1}H NMR (D_2O) δ 1.45 (m, 4 H), 1.70 (m, 8 H), 3.01 (m, 8 H). Anal. Calcd. for $C_{10}H_{28}Cl_3N_3$: C, 40.48; H, 9.51; N, 14.16; Cl, 35.85. Found: C, 40.63; H, 9.44; N, 14.16; Cl, 35.70.

1,7,13-Trimesitylenesulfonyl-1,7,13-triazatridecane (89). A solution of **84** (3.47 g, 9.4 mmol) in 1 N NaOH (30 mL), 2-mesitylenesulfonyl chloride (4.52 g, 20.7 mmol) in CH_2Cl_2 were combined and worked up by the method of **63**. Column chromatography (3:2 hexane/EtOAc) gave 6.44 g (93%) **89** as a white solid: ^{1}H NMR δ 1.16 (m, 4 H), 1.39 (m, 8 H), 2.30 (s, 3 H), 2.31 (s, 6 H), 2.57 (s, 6 H), 2.62 (s, 12 H), 2.81 (2 t, 4 H, J = 7), 3.10 (t, 4 H), 4.49 (t, 2 H, J = 7), 6.95 (s, 2 H), 6.97 (s, 4 H). High resolution mass spectrum ($C_{37}H_{55}N_3O_6S_3$): M+H 734.3331; found 734.3351.

2,8,14-Trimesitylenesulfonyl-2,8,14-triazapentadecane (99). Sodium hydride (80%, 0.207 g, 6.9 mmol), a solution of **89** (1.581 g, 2.16 mmol) in DMF (30 mL) and iodomethane (0.674 g, 300 mL, 4.75 mmol) were combined and worked up by the method of **65**. Column chromatography (5:2 hexane/EtOAc) provided 1.51 g of **99** (92%) as an oil: ^{1}H NMR δ 1.06-1.18 (m, 4 H), 1.40-1.52 (m, 8 H), 2.29 (s, 9 H), 2.59 (s,

18 H), 2.66 (s, 6 H), 3.03-3.14 (m, 8 H), 6.95 (s, 6 H). Anal. Calcd. for $C_{39}H_{59}N_3O_6S_3$: C, 61.47; H, 7.80; N, 5.51. Found: C, 61.54; H, 7.79; N, 5.51.

2,8,14-Triazapentadecane Trihydrochloride (46). HBr in HOAc (30%, 30 mL) and a solution of **99** (1.48 g, 1.94 mmol) and phenol (5.2 g, 55.3 mmol) were combined and worked up by the method of **1**. Recrystallization from aqueous EtOH produced 480 mg (76%) **46** as needles: ^{1}H NMR (D_2O) δ 1.4-1.5 (quintet, 4 H), 1.7-1.8 (quintet, 8 H), 2.7 (s, 6 H), 3.05 (t, 8 H, J = 7). Anal. Calcd. for $C_{12}H_{32}N_3Cl_3$: C, 44.38; H, 9.93; N, 12.94. Found: C, 44.33; H, 9.90; N, 12.89.

3,9,15-Trimesitylenesulfonyl-3,9,15-triazaheptadecane (100). Sodium hydride (80%, 0.52 g, 17.4 mmol), a solution of **89** (3.2 g, 4.36 mmol) in DMF (20 mL) at 0 °C. and iodoethane (1.5 g, 9.6 mmol) were combined and worked up by the method of **65**. Column chromatography (4:1 hexane/EtOAc) gave 2.91 g (85%) of **100** as a white solid: mp = 60-62 °C. ^{1}H NMR δ 1.01 (t, 6 H, J = 7), 1.08 (m, 4 H), 1.42 (m, 8 H), 2.29 (s, 9 H), 2.57 (s, 6 H), 2.58 (s, 12 H), 3.07 (t, 4 H, J = 7), 3.11 (t, 4 H, J = 7), 3.17 (quartet, 4 H), 6.92 (s, 6 H). Anal. Calcd. for $C_{41}H_{63}N_3O_6S_3$: C, 62.32; H, 8.04; N, 5.32; S, 12.17. Found: C, 62.40; H, 8.08; N, 5.25; S, 12.07.

3,9,15-Triazaheptadecane Trihydrochloride (47). A solution of **100** (2.9 g, 3.67 mmol) and phenol (3.25 g, 34.5 mmol) in $CHCl_3$ (27 mL) and 30% HBr in HOAc (20 mL) were combined and worked up by the method of **1**. Recrystallization from aqueous EtOH provided 1.0 g (77%) of **47** as white crystals: ^{1}H NMR (D_2O) δ 1.28 (t, 6 H, J = 7), 1.45 (m, 4 H), 1.73 (m, 8 H), 3.08 (m, 12 H). Anal. Calcd. for $C_{14}H_{36}Cl_3N_3$: C, 47.66; H, 10.28; N, 11.91; Cl, 30.15. Found: C, 47.69; H, 10.24; N, 11.96; Cl, 30.08.

4,10,16-Trimesitylenesulfonyl-4,10,16-triazanonadecane (101). NaH (80%, 198 mg, 6.6 mmol), a solution of **89** (1.512 g, 2.06 mmol) in DMF (40 mL), and 1-iodopropane were combined and worked up by the method of **65**. Column chromatography (7:2 hexane/EtOAc) provided 1.61 g (95%) of **101** as a thick oil: ^{1}H NMR δ 0.75 (t, 6 H, J = 7), 1.02-1.14 (m, 4 H), 1.36-1.52 (m, 12 H), 2.30 (s, 9 H), 2.60

(s, 18 H), 3.02-3.16 (m, 12 H), 6.95 (s, 6 H). Anal. Calcd. for $C_{43}H_{67}N_3O_6S_3$: C, 63.12; H, 8.25; N, 5.14. Found: C, 63.21; H, 8.23; N, 5.04.

4,10,16-Triazanonadecane Trihydrochloride (48). HBr in HOAc (30% , 30 mL) and a solution of **101** (1.58 g, 1.93 mmol) and phenol (5.2 g, 55.3 mmol) in CH_2Cl_2 (100 mL) were combined and worked up by the method of **1.** Recrystallization from aqueous EtOH gave 579 g (79%) of **48** as plates: ^{1}H NMR (D_2O) δ 0.95 (t, 6 H, J = 7), 1.38-1.49 (m, 4 H), 1.62-1.77 (m, 12 H), 2.95-3.05 (m, 12 H). Anal. Calcd. for $C_{16}H_{40}Cl_3N_3$: C, 50.46; H, 10.59; N, 11.03. Found: C, 50.49; H, 10.55; N, 11.07.

***N*^{1}-Triphenylmethyl-1,3-diaminopropane (106).** A solution of triphenylmethyl chloride (6.97 g, 25 mmol) in dry CH_2Cl_2 (100 mL) was added dropwise to a rapidly stirred solution of 1,3-diaminopropane (9.86 g, 133 mmol) in dry CH_2Cl_2 (100 mL). After stirred at room temperature for 2 days, the obtained suspension was worked up by adding 1 N NaOH (50 mL) to get two clear phases with the aqueous layer pH = 14, which was then extracted with $CHCl_3$ (3 x 50 mL). Combined organic portion was washed with dist. H_2O (100 mL) and brine (100 mL). The solvent was removed by rotvap and purification with 3% NH_4OH/MeOH gave 6.32 g (80%) of **106** a white solid: ^{1}H NMR δ 1.35-1.65 (m, 6 H), 2.18 (t, 2 H, J = 7), 2.77 (s, 1 H), 7.13-7.24 (m, 9 H), 7.44-7.46 (m, 6 H). Anal. Calcd. for $C_{22}H_{24}N_2$: C, 83.50; H, 7.64; N, 8.85. Found C, 83.42; H, 7.67; N, 8.86.

***N*^{1}-Triphenylmethyl-1,4-diaminobutane (107).** A solution of triphenylmethyl chloride in CH_2Cl_2 (500 mL) was added dropwise to a rapidly stirred solution of solution of 1,4-diaminobutane (96.47 g, 1.094 mol) in CH_2Cl_2 (1.1 L) over a period of 2 h. The reaction was stirred at room temperature for 3 days and was worked up by the method of **106** to give a quantitative yield of **107** as an oil which was used directly in the next step: ^{1}H NMR δ 1.39-1.54 (m, 7 H), 2.12 (t, 2 H, J = 7), 2.62 (t, 2 H, J = 7), 7.13-7.28 (m, 9 H), 7.41-7.47 (m, 6 H).

***N*^{1}-Mesitylenesulfonyl-*N*^{3}-triphenylmethyl-1,3-diaminopropane (108).** Mesitylenesulfonyl chloride (5.25 g, 24 mmol) and **106** (6.30 g, 20 mmol) in CH_2Cl_2 (30 mL) and 1 N NaOH (27 mL) were combined and worked up by the method of **63**. Column chromatography (7:2 hexane/EtOAc) gave 8.37 g (84%) of **108** as a white solid: ^{1}H NMR δ 1.56-1.66 (m, 3 H), 2.17 (t, 2 H, *J* = 7), 2.29 (s, 3 H), 2.60 (s, 6 H), 3.08 (quartet, 2 H), 5.25 (br t, 1 H), 6.93 (s, 2 H), 7.15-7.28 (m, 9 H), 7.35-7.39 (m, 6 H). Anal. Calcd. for $C_{31}H_{34}N_2O_2S$: C, 74.67; H, 6.87; N, 5.62. Found: C, 74.62; H, 6.89; N, 5.54.

***N*^{1}-Mesitylenesulfonyl-*N*^{4}-triphenylmethyl-1,4-diaminobutane (109).** Mesitylenesulfonyl chloride (3.1 g, 14 mmol) and **107** (3.39 g, 10.3 mmol) in CH_2Cl_2 (20 mL) and 1 N NaOH (15 mL) were combined and worked up by the method of **63**. Column chromatography (3:1 hexane/EtOAc) furnished 3.79 g (72%) of **109** as a white solid: ^{1}H NMR δ 1.41-1.50 (m, 5 H), 2.03-2.08 (t, 2 H, *J* = 7), 2.27 (s, 3 H), 2.62 (s, 6 H), 2.87 (quartet, 2 H), 4.41 (br t, 1 H), 6.93 (s, 2 H), 7.15-7.28 (m, 9 H), 7.40-7.44 (m, 6 H). Anal. Calcd. for $C_{32}H_{36}N_2O_2S$: C, 74.97; H, 7.08; N, 5.46. Found: C, 74.71; H, 7.12; N, 5.51. -

***N*-Propylmesitylenesulfonamide (64).** Mesitylenesulfonyl chloride (12.0 g, 55 mmol) and propylamine (2.96 g, 50 mmol) in CH_2Cl_2 (60 mL) and 1 N NaOH (60 mL) were combined and worked up by the method of **63**. Column chromatography (3:1 hexane/EtOAc) afforded 8.44 g (85%) of **64** as crystalline solid: mp 53-54 °C; ^{1}H NMR δ 0.86 (t, 3 H, *J* = 7), 1.44-1.51(m, 2 H), 2.30 (s, 3 H), 2.64 (s, 6 H), 2.86 (quatet, 2 H), 4.40 (br t, 1 H), 6.96 (s, 2 H). Anal. Calcd. for $C_{12}H_{19}NO_2S$: C, 59.72; H, 7.93; N, 5.80. Found: C, 59.69; H, 7.88; N, 5.80.

***N*-(3-Bromopropyl)-*N*-propylmesitylenesulfonamide (68).** Sodium hydride (60%, 0.34 g, 8.4 mmol), **64** (1.7 g, 7.0 mmol) and 1,3-dibromopropane (16.96 g, 84 mmol) in DMF (30 mL) were combined and worked up by the method of **65**. Column chromatography (6:1 hexane/EtOAc) produced 1.82 g (80%) **68** as an oil: ^{1}H NMR δ

0.79 (s, 3 H), 1.48-1.56 (m, 2 H), 2.04-2.10 (m, 2 H), 2.30 (s, 3 H), 2.60 (s, 6 H), 3.09-3.14 (m, 2 H), 3.29-3.36 (m, 4 H). Anal. Calcd. for $C_{15}H_{24}BrNO_2S$: C, 49.72; H, 6.68; N, 3.87. Found C, 49.97; H, 6.76; N, 3.83.

***N*-(4-Bromobutyl)-*N*-propylmesitylenesulfonamide (69).** Sodium hydride (60%, 0.70 g, 17.4 mmol), **64** (3.5 g, 14.5 mmol), and 1,4-dibromobutane (37.6 g, 174 mmol) in DMF (40 mL)were combined and worked up the method of **63**. Excess 1,4-dibromobutane was removed by a Kugelrohr apparatus under high vacuum. Column chromatography (7:1 hexane/EtOAc) produced 5.21 g (95%) of **69** as an oil: ^{1}H NMR δ 0.79 (t, 3 H, J = 7), 1.46-1.54 (m, 2 H), 1.64-1.78 (m, 4 H), 2.30 (s, 3 H), 2.60 (s, 6 H), 3.11 (t, 2 H, J = 7.5), 3.21 (t, 2 H, J = 7), 3.31(t, 2 H, J = 7), 6.93 (s, 2 H). Anal. Calcd. for $C_{16}H_{26}BrNO_2S$: C, 51.06; H, 6.96; N, 3.72. Found: C, 51.17; H, 6.95; N, 3.74.

6,10-Bis(mesitylenesulfonyl)-1-triphenylmethyl-1,6,10-triazatrideane (110). Sodium hydride (60%, 0.24 g, 5.96 mmol) was added in portion into a solution of **109** (2.55 g, 4.97 mmol) in DMF (40 mL) at 0 °C. The suspension was stirred for 20 min at 0 °C, and a solution of **68** (1.80 g, 4.97 mmol) in DMF (20 mL) was introduced. The mixture was stirred at room temperature for 24 h and then worked up following the method of **65**. Column chromatography (20:1 toluene/EtOAc) produced 3.72 g (94%) of **110** as an oil: ^{1}H NMR δ 0.73 (t, 3 H, J = 7), 1.26-1.70 (m, 8 H), 2.01 (t, 2 H, J = 7), 2.26 (s, 3 H), 2.27 (s, 3 H), 2.54 (s, 12 H), 2.96-3.04 (m, 8 H), 6.90 (s, 4 H), 7.18-7.45 (m, 15 H). Anal. Calcd. for $C_{47}H_{59}N_3O_4S_2$: C, 71.09; H, 7.49; N, 5.29. Found C, 71.35; H, 7.53; N, 5.18.

5,10-Bis(mesitylenesulfonyl)-1-triphenylmethyl-1,5,10-triazatrideane (111). Sodium hydride (60%, 0.22 g, 5.42 mmol), **108** (2.25 g, 4.52 mmol) in DMF (40 mL), and **69** (1.70 g, 4.52 mmol) in DMF (20 mL) were reacted and worked up following the method of **110**. Column chromatography (25:1 toluene/EtOAc) produced 2.87 g (80%) **111** as an oil: ^{1}H NMR δ 0.75 (t, 3 H, J = 7), 1.41-1.46 (m, 9 H), 1.97 (t, 2 H, J = 7), 2.26 (s, 6 H), 2.53 (s, 6 H), 2.56 (s, 6 H), 3.04(t, 2 H, J = 7), 3.10-3.15 (m, 6 H), 6.88 (s, 2 H),

6.90 (s, 2 H), 7.15-7.38 (m, 15 H). Anal. Calcd. for $C_{47}H_{59}N_3O_4S_2$: C, 71.09; H, 7.49; N, 5.29. Found C, 71.16; H, 7.46; N, 5.33.

6,11-Bis(mesitylenesulfonyl)-1-triphenylmethyl-1,6,11-triazatetradeane (112). Sodium hydride (60%, 0.12 g, 2.90 mmol), **109** (1.24 g, 2.42 mmol) in DMF (30 mL), and **69** (0.91 g, 2.42 mmol) in DMF (10 mL) were combined worked up by the method of **110**. Column chromatography (25:1 toluene/EtOAc) produced 1.67 g (85%) **112** as an oil: ^{1}H NMR δ 0.74 (t, 3 H, J = 7), 1.35-1.45 (m, 11 H), 2.00 (t, 2 H, J = 7), 2.25 (s, 3 H), 2.28 (s, 3 H), 2.55 (s, 6 H), 2.56 (s, 6 H), 2.98-3.08 (m, 8 H), 6.90 (s, 2 H), 6.91 (s, 2 H), 7.15-7.44 (m, 15 H). Anal. Calcd. for $C_{48}H_{61}N_3O_4S_2$: C, 71.34; H, 7.61; N, 5.20. Found C, 71.22; H, 7.64; N, 5.10.

***N*^{1}-Propylnorspermidine Trihydrochloride (26).** HBr (30% in HOAc, 30 mL), **91** (1.14 g, 1.58 mmol) and phenol (6.4 g, 68.0 mmol) in CH_2Cl_2 (25 mL) were reacted, and product was isolated by the method of **1** to give 87.0 mg (20%) of **26** as white powder: ^{1}H NMR (D_2O) δ 0.98 (t, 3 H, J = 7), 1.64-1.77 (m, 2 H), 2.05-2.18 (m, 4 H), 3.01-3.22 (m, 10 H). Anal. Calcd. for $C_9H_{26}Cl_3N_3$: C, 38.24; H, 9.27; N, 14.87. Found C, 38.15; H, 9.32; N, 14.75.

***N*^{1}-Propylspermidine Trihydrochloride (33).** HBr (30% in HOAc, 45 mL), **110** (3.70 g, 4.66 mmol) and phenol (8.4 g, 89.0 mmol) in CH_2Cl_2 (50 mL) were reacted, and the product was isolated by the method of **1** to give 1.02 g (74%) **33** as plates: ^{1}H NMR (D_2O) δ 0.98 (t, 3 H, J = 7), 1.66-1.79 (m, 6 H), 2.06-2.17 (m, 2 H), 3.01-3.19 (m, 10 H). Anal. Calcd. for $C_{10}H_{28}Cl_3N_3$: C, 40.48; H, 9.51; N, 14.16. Found C, 40.55; H, 9.45; N, 14.18.

***N*^{8}-Propylspermidine Trihydrochloride (34).** HBr (30% in HOAc, 35 mL), **111** (2.85 g, 3.59 mmol) and phenol (6.5 g, 69 mmol) in CH_2Cl_2 (30 mL) were.reacted, and the product was isolated by the method of **1** to give 810 mg (76%) **34** as plates: ^{1}H NMR (D_2O) δ 0.98 (t, 3 H, J = 7), 1.66-1.79 (m, 6 H), 2.01-2.12 (m, 2 H), 2.99-3.14

(m, 10 H). Anal. Calcd. for $C_{10}H_{28}Cl_3N_3$: C, 40.48; H, 9.51; N, 14.16. Found C, 40.52; H, 9.52; N, 14.09.

***N*^{1}-Propylhomospermidine Trihydrochloride (39).** HBr (30% in HOAc, 35 mL), **112** (1.65 g, 2.0 mmol) and phenol (3.6 g, 38 mmol) in CH_2Cl_2 (20 mL) were reacted, and the product was isolated by the method of **1** to give 268 mg (43%) of **39** as plates: ^{1}H NMR (D_2O) δ 0.98 (t, 3 H, J = 7), 1.66-1.80 (m, 10 H), 2.99-3.11 (m, 10 H). High resolution mass spectrum ($C_{11}H_{27}Cl_3N_3$): M+H 202.2283; Found 202.2296

***N*-Bis(4-phthalimidobutyl)mesitylenesulfonamide (113).** Sodium hydride (1.6 g, 40 mmol), was added to a solution of **77** (2.72 g, 13.5 mmol) in DMF (60 mL) at 0 °C. After the mixture was stirred at 0 °C for 30 min, *N*-(4-bromobutyl)phthalimide (11.51 g, 40 mmol) in DMF (20 mL) was introduced. The mixture was stirred at room temperature for 1 h and at 60 °C overnight. Following workup by procedure of **65**, column chromatography (25:1, $CHCl_3$/acetone) gave 3.77 g (46%) **113** as a white powder: ^{1}H NMR δ 1.51-1.54 (m, 8 H), 2.18 (s, 3 H), 2.57 (s, 6 H), 3.18-3.24 (m, 4 H), 3.55-3.60 (m, 4 H), 6.86 (s, 2 H), 7.69-7.25 (m, 4 H), 7.82-7.85 (m, 4 H). Anal. Calcd for $C_{33}H_{35}N_3O_6S$+ 0.6 H_2O: C, 64.71; H, 5.96; N, 6.86. Found: C, 64.57; H, 5.80; N, 6.98. High resolution mass spectrum ($C_{33}H_{35}N_3O_6S$): M+H 602.2324. Found 602.2320.

***N*-Bis(4-aminobutyl)mesitylenesulfonamide (82).** Hydrazine monohydride (0.82 g) was added to a suspension of **113** (3.5 g) in abs. EtOH (100 ml) at room temperature. After mixture suspension was stirred at 65 °C for 24 h, the white solid was filtered and washed with abs.EtOH (2 x 10 mL). The combined filtration was concentrated and purified by flash column chromatography with MeOH/NH_4OH (6:1). **82** (1.50 g, 76%) was produced as a thick oil: ^{1}H NMR δ 1.37 (quintet, 4 H), 1.52 (quintet, 4 H), 2.30 (s, 3 H), 2.54 (t, 4 H, J = 7), 2.58 (s, 6 H), 3.20 (t, 4 H, J = 7), 7.04 (s, 3 H). Anal. Calcd for $C_{17}H_{31}N_3O_2S$ + 0.7 H_2O: C, 57.66; H, 9.22; N, 11.87; S, 9.01. Found: C, 57.61; H, 9.18; N, 11.53; S, 8.98.

Homospermidine Trihydrochloride (36). HBr in HOAc (30% in HOAc, 30 mL), **82** (1.50 g, 4.39 mmol) and phenol (4.49 g, 48 mmol) in CH_2Cl_2 (20 mL)were reacted and worked up following the procedure of **1.** Recrystallization from aqueous EtOH gave **36** (0.86 g, 73%) as white crystals: ^{1}H NMR (D_2O) δ 1.73-1.80 (m, 8 H), 3.03-3.14 (m, 8 H). Anal. Calcd for $C_8H_{24}Cl_3N_3$: C, 35.77; H, 9.00; N, 15.64; S, 39.59. Found: C, 35.88; H, 8.91; N, 15.68; S, 39.48.

Cell Culture And Biological Assays

Cell culture materials were obtained from Sigma Chemical Co. RPMI-1640 medium, fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 3-(N-morpholino)-propanesulfonic acid (MOPS) were obtained from Gibco (Grand Island, NY). Cell culture flasks, 25, 75 and 150 cm^2, were bought from Corning (Coring, NY). 10 mL side-arm flask, rubber stoppers and polypropylene wells used in enzyme assays were obtained from Knotes Glass Company (Vineland, New Jersey). Cell numbers were determined by electronic particle analysis (Coulter Counter, Model Z_F, Coulter Electronics, Hialeah, FL). ^{3}H-spermidine, (acetyl-1-^{14}C)-Acetyl Coenzyme A and Biofluor liquid scintillation fluid were bought from New England Nuclear Research (Boston, MA). L-(carboxyl-^{14}C)-ornithine and S-adenosyl-L-(carboxyl-^{14}C)methionine were purchased from Amersham Corp. (Arlington Hts., IL). All other organic reagents and solvents were purchased from Aldrich Chemical Co. or Fisher Scientific Co. HPLC solvents were obtained from Fisher Scientific Co. The solid phase extraction columns (SPE-3 mL-500 mg) were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Murine L1210 leukemia cells were obtained from the American Type Tissue Corporation and maintained in logarithmic growth as suspension cultures in RPMI-1640 medium containing 16 mM (HEPES), 8 mM (MOPS), 1 mM aminoguanidine (diamino oxidase inhibitor), 1 mM L-glutamine and 10% fetal bovine serum. Cells were grown in

25 cm^2 tissue culture flasks (Corning) in a total volume of 10 mL under a humidified 5% CO_2 atmosphere at 37 °C.

Human melanoma cells MALME-3M were obtained from the American Type Tissue Corporation and maintained as monolayer culture growth in RPMI 1640 containing 1 mM aminoguanidine (diamine oxidase inhibitor), 1 mM L-glutamine and 10% NuSerum. Cells were grown in 25 cm^2 tissue culture flasks with vented cap (Corning) in a total volume of 10 mL under a humidified 5% CO_2 atmosphere at 37 °C.

Spermidine and spermine were obtained as hydrochloride salt (Sigma). *N*-(3-aminopropyl)-1,3-propanediamine [norspermidine (**22**)] was converted to its trihydrochloride salt and recrystallized from aqueous ethanol. Besides the compounds synthesized in this study, additional polyamine derivatives were also studied for activity against L1210 cells lines. The compounds included: (1) tetraamine analogs (Bergeron 1994): DENSPM (**3**), DPNSPM(**4**), MESPM (**8**), DESPM (**9**), DPSPM (**10**), MEHSPM (**13**), DEHSPM (**14**), ETBHSPM (**16**), DTBHSPM (**17**), DE(3,4,4) (**18**), DE(4,3,4) (**19**) and DE(5,4,5) (**21**); (2) triamine analogues: MENSPD (**24**), DENSPD (**25**), N^1-ethylspermidine [MESPD(N^1) (**30**)], N^8-ethylspermidine [MESPD(N^8) (**31**)], N^1,N^8-diethylspermidine [DESPD (**32**)], N^1,N^8-dipropylspermidine [DPSPD (**35**)]. These compounds were generously provided by Dr. James McManis, Dr. Charlie Z.R. Liu, Dr. Guowei Yao, Mr. Timotyl Vinson and Mr. Sam Algee of the Department of Medicinal Chemistry in the College of Pharmacy at the University of Florida.

<u>IC_{50} Determinations of L1210 cells</u>

The trihydrochloride or tetrahydrochloride salts of the polyamine derivatives were diluted in sterile water, filtered through 0.2 μM filters, and stored at 4 °C. The L1210 cells were treated while in logarithmic growth (5 x10^4 cells/mL). Cells from stock cultures were seeded in fresh medium and incubated in the presence of polyamine derivative at concentrations from 0.01 to 100.00 μM for 48 h. Cell samples were

removed after 48 h and counted via electronic particle analysis on a Coulter Counter. The cells were reseeded as 5 $x10^4$ cells/mL in fresh medium with the presence of corresponding concentration of polyamine derivatives. After an other 48 h, the cells were removed and counted as before. The percentage of control growth was determined as follows:

$$\% \text{ of growth} = \frac{\text{Final treated cell no. - initial inoculum}}{\text{Final untreated cell no. - initial inoculum}} \times 100$$

The IC_{50} is defined as the concentration of compound necessary to reduce cell growth to 50% of control growth after defined intervals of exposure. In this dissertation, IC_{50} is determined at 48 h and 96 h.

Competitive Uptake Determination in L1210 cells

Polyamine analogues were compared with respect to their ability to compete with [^{3}H] labeled spermidine for uptake in L1210 leukemia cell in vitro. L1210 cells from serially passaged stock cultures were cooled in ice-slur for 10 min, centrifuged at 900 g at 0 °C for 10 min and resuspended in complete culture medium with 1 mM aminoguanidine, to a concentration of approximately 7 x 10^6 cells/mL. The exact concentration was recorded. The reaction was carried out in polyprolene tubes (Sarstedt-15 mL). To prepare the radiolabeled probe, 10 µL stock ^{3}H-spermidine (NEC) was added to 1.475 mL complete media containing 1 mM aminoguanidine. To this was added 15 µL of a 10 mM spermidine solution, thus yielding a 100 µM spermidine solution. To prepare each tube for the addition of cells, inhibitor and ^{3}H-spermidine were added and the volume adjusted to 500 µL with complete media. Three concentrations of inhibitor were used for each assay: 10 µM, 25 µM and 50 µM. For compounds with high Ki (> 100 µM) higher inhibitor concentrations: 100 µM, 250 µM and 500 µM are used in the assay for more accurate results.

Polyamine Pool Analysis

Preparation of cell culture samples

While in logarithmic growth, cells were treated with the polyamine derivatives. At the end of the treatment period, cell suspensions were sampled, washed twice in cold fresh RPMI-1640 medium (2 x 10 mL) and centrifuged. The supernatant was carefully removed and the cells resuspended in 0.6 M perchloric acid (1 x 10^7 cells/mL). The cells were then freeze-fractured by four successive freeze-thawings using liquid nitrogen. When necessary, cell lysates were stored at -20 °C until analysis by HPLC analysis.

HPLC Analysis

A 100-µL aliquot of the solubilized polyamine perchlorates was reacted with 400 µL dansyl chloride (5 mg/ml in acetone), sodium carbonate (0.12 g) and an appropriate internal standard. i.e. 30 µL of 1.25 x 10^{-4} M 1,6-diaminohexane (DHX). After vortexing, the reaction mixture was heated at 70 °C for 20 min and again vortexed and cooled to room temperature (20 min). An aqueous proline solution (100 µL of 250 mg/ml) was added to quench the excess dansyl chloride and allowed to react at room temperature for 10 min. The dansylated polyamines were transferred using 25% aqueous CH_3CN (2 x 500 µL) to a solid phase extraction column which had been prepared by successive rinses of MeOH and aqueous CH_3CN. The column was aspirated, rinsed with 25% aqueous CH_3CN (2 x 2.5 mL), the sample eluted with MeOH (2 x 2 mL), and the eluent volume adjusted to 5 mL with MeOH. Part of the sample was analyzed by the Rainin Instrument Company HPLC system, which incorporates control and data acquisition by a microcomputer using Rainin Dynamax software and hardware. Automatic sample injection is performed via a 20-µL fixed sample loop on the Rheodyne injection valve of a Gilson autosampler/diluter. Fluorescence detection by a Hewlett-Packard HP 1046A programmable detector was employed (ex = 228 nm; em = > 470 nm). A Beckman C-18 column (4.6 mm x 150 mm, 5-µm spherical packing) follows a Rheodyne in-line filter and a Rainin guard column. The solvent gradient program

consists of an initial 3-min isocratic portion with 45% aqueous CH_3CN followed by a linear gradient to MeOH at 18 min.

Enzyme Assay of ODC

ODC activity was determined according to the modified procedures of Seely and Pegg on the basis of quantitation of $^{14}CO_2$ released from [^{14}C]carboxyl-labeled L-ornithine (1983). Three flasks (T150) of cells were treated for each compound tested, and each flask was sampled in triplet. DEHSPM treated cells were used as a positive control in each assay.

Preparation

The L1210 cells (5 x10^5 cells/mL) were treated with 1 μM polyamine analogues for 4 h. After counted on Coulter Counter and cooled in ice-slur (10 min), cells were centrifuged (1200 rpm, 10 min). The pellet was washed with incomplete media (RPMI 1640, 2 x 50 mL). The cells were centrifuged (1200 rpm) for 5 min followed each wash. Certain volume of Tris buffer A (10 mL of 0.5 M Tris-HCl, 40 μL of 0.5 EDTA, 38.7 mg dithiothreitol and 20 mg pyridoxyl phosphate in a total volume of 200 mL, pH = 7.4) was added to the pellet to make a 10^6 cells/250 μL suspension. The cells were disrupted by ultrasound and 1 mL of the supernatant was centrifuged (20,000 rpm, 3.0 min) at 4 °C. The obtained supernatant was saved for the assay. A substrate solution was made up containing 965.2 μL of Tris buffer B (1 mM L-ornithine in Tris buffer A) and 34.8 μL of L-[1-^{14}C]ornithine (40-60 mCi/mmol).

Enzyme assay

An assay mixture is made up containing 50 μL of substrate solution and 200 μL of the enzyme suspension making a total volume of 0.25 mL. The reaction was carried out in 10 mL side-arm flask closed with rubber stoppers carrying polypropylene wells containing 0.2 mL of 1 M benzethonium hydroxide and a piece of filter paper (Whatman 3 MM, 0.4 x 2 cm^2). The assay was started by the addition of enzyme suspension, and

the flasks were incubated at 37 °C for 30 min in a shaking water bath. The reaction was then stopped by the injection of 0.3 mL of 0.5 M sulfuric acid through the rubber cap. The acid released $^{14}CO_2$ from the assay medium, and, after a further 15 min at 37 °C to ensure complete absorption of the $^{14}CO_2$ in the benzethonium hydroxide, the well was removed, placed in 10 mL of toluene-based scintillation fluid, and counted in Beckman liquid scintillation counter.

Enzyme Assay of AdoMet

AdoMet activity was determined according to the modified procedures of Pegg and Pösö on the basis of quantitation of $^{14}CO_2$ released from S-adenosyl-L-[carboxyl-^{14}C]methionine (1983). Three (T150) of L1210 cells were treated for each compound tested, and each flask was sampled in triplet. DEHSPM treated cells were used as positive control in each assay.

Preparation

L1210 cells were treated with 1 μM of drug 6 h ahead of assay. The enzyme solution was prepared as described in ODC assay. The substrate solution was made up containing 742 μL 10 mM putrescine solution in Tris buffer A, 1.3 mg S-adenosyl-L-methionine, 8 μL 1 N NaOH and 250 μL S-adenosyl-L-[carboxyl-^{14}C]methionine (50 mCi/mmole) to give a final volume of 1.00 mL.

Enzyme assay

The procedure was as same as that for ODC assay except that after the reaction was terminated, a further 30 min of incubation was needed to ensure complete absorption of $^{14}CO_2$.

Enzyme Assay of SSAT

Spermidine/spermine N^1-acetyltransferase (SSAT) activity was determined according to the modified procedure based on quantitation of the production of [^{14}C]-N^1-

acetylspermidine formed by acetylation of spermidine with [^{14}C] acetyl coenzyme A according to the method of Libby et al. (1989b).

Cells treated with DENSPM were positive controls. L1210 cells were treated with polyamine analogues (10 μM for triamine analogues and 2 μM for tetraamine analogues) 48 h ahead of assay. All enzyme activity assays were determined using an enzyme extract obtained by sonication of treated and untreated cells in medium containing 5 mM HEPES (pH 7.6) and 1 mM dithiothreitol. The cytosol obtained following a 4 h centrifugation at 12,000 rpm in a microcentrifuge (Allied, Fisher Scientific, Model 59A) was used as the source of enzyme.

In addition to measuring SSAT, this assay also detects a nuclear acetyltransferase, histon acetylase, and possibly other cellular acetyltransferases that are capable of acetylating polyamines. It is presumed, the basis of findings made in other cell systems where enzyme-specific activity has been used that the induced enzyme activity is entirely that of SSAT.

Toxicity Study

CD-1 female mice with 10-12 weeks of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were selected at random for experiments. For each acute toxicities, the polyamine analogues were administered in a single i.p. injection to groups of five to six animals. All survivors were further observed for 10 days to assess late onset of toxicity from the single acute dose. In the chronic toxicity regimen, mice were administered polyamine analogue in three i.p. dose per day (t.i.d.) for six days, a total of eighteen doses per animal. Appetite, weight and overall appearance were monitored daily. Animals were observed for 10 days following the final dose, at which time the final score was registered. At least three test groups of 5-6 animals each, representing three different dose levels, were evaluated for each analogue tested. These

dose levels were chosen so that at least two groups presented with lethalities, one with a high fraction of lethalities (>0.50, but <1.00).

IC_{50} Determination of MALME-3 Cells

Cells were seeded at 2 x 10^5 cells per T-25 flask (8 x $10^3/cm^2$) in a total volume of 10 mL. Cells were incubated for 24 h before treatment. Cells were treated with DE(4,5) and incubated for 96 h without media change. Cells were harvested by trypsinization and cell suspensions were counted using electronic particle counting (Model Z_F coulter counter) and confirmed periodically by hemocytometer measurements.

CHAPTER 5
BIOLOGICAL ACTIVITIES

In summarizing the biological properties of the polyamine analogues, the results of triamines and tetraamines will be discussed separately. In each series, the results will be separated into three sets of measurements: (1) the 48 and 96 h IC_{50} values against L1210 cells and the corresponding K_i values for the polyamine transport apparatus, Table 5-1 (tetraamines) and Table 5-4 (triamines). (2) the effect on polyamine pools, Table 5- 2 (tetraamines) and Table 5-5 (triamines). (3) the impact on ornithine decarboxylase, S-adenosylmethionine decarboxylase, and spermidine/spermine N^1-acetyltransferase activities, Table 5-3 (tetraamines) and Table 5-6 (triamines).

Biological Evaluation of Tetraamine Analogues

The tetraamines analogues are arranged into the following sets of compounds: norspermines, spermines, homospermines and homospermine homologues. The K_i value of spermine has been reported as 9.2 μM in previous study (Porter & Bergeron 1983). In this study, from our currently cultured L1210 cell line, a Ki value of 0.7 μM was measured as an average of three measurements under different inhibitor concentrations.

Antiproliferative Activity - IC_{50} of L1210 Cells

As shown in Table 5-1, whether or not both terminal nitrogens of norspermine were symmetrically substituted with methyl, ethyl, or isopropyl groups or had a single ethyl fixed to one of the terminal nitrogens was insignificant relative to the 48 and 96 h IC_{50}'s. All of the 48 h IC_{50} values were in excess of 100 μM and the 96 h IC_{50} values

were all around 2 μM. When the terminal nitrogens of norspermine were substituted with n-propyl, the 95 h IC_{50} activity significantly decreased.

Unlike the norspermines, two of the spermine analogues, N^1,N^{12}-di-*n*-propylspermine (DPSPM) and DESPM, had a significant effect on cell growth at 48 h. The IC_{50} concentrations ranged from 3 to > 100 μM, with DPSPM < DESPM < N^1-ethylspermine (MESPM) < N^1,N^{12}-dimethylspermine (DMSPM). There was little difference in the 96 h IC_{50} values when the terminal nitrogens of spermine were symmetrically substituted with either a single methyl, ethyl, or propyl group or when a single ethyl was fixed to one of the terminal nitrogens. Compared to DENSPM, its branched analogue DECroNSPM showed very similar IC_{50} activities, > 100 μM at 48 h and 0.8 μM at 96 h. While the 96 h IC_{50} of DECroSPM fell in the same range as the other SPM analogues, at 48 h, with IC_{50} of 2.5 μM, DECroSPM was more than ten times active than its linear analogue DESPM.

There are considerable differences between the various alkylated homospermines at both 48 and 96 h. Unlike the norspermines, all of which had 48 h IC_{50}'s > 100 μM, the corresponding homospermine analogues, except N^1,N^{14}-dimethylhomospermine (DMHSPM) had 48 h IC_{50}'s < 25 μM. The most notable differences in activity were between the diethyl and dimethyl compounds and between ETBHSPM and N^1,N^{14}-di-*tert*-butylhomospermine (DTBHSPM). The most active of all the homospermines is DEHSPM at 48 h with a 0.2 mM IC_{50}, 70 times more effective than MEHSPM, 100 times more active than N^1,N^{14}-diisopropylhomospermine (DIPHSPM) and at least 500 times more active than DMHSPM. While replacement of a single ethyl group of DEHSPM with a *tert*-butyl group, N^1-ethyl-N^{14}-*tert*-butylhomospermine (ETBHSPM), resulted in a compound very similar in 48 h activity to DEHSPM. In contrast, the DTBHSPM compound had an IC_{50} > 100 μM. It would seem that one sterically unhindered end of the homospermine analogues is required for significant 48 h activity. At 96 h, although there are differences in potency among the various homospermine analogues, they were not as impressive as the

differences at 48 h. The most notable difference, 100 fold, occurred between the *tert*-butyl substituted homospermines ETBHSPM and DTBHSPM.

The outcome of holding the terminal alkyl groups and the overall length of the polyamine constant while changing the distance between the central and terminal nitrogens is interesting at an IC_{50} level. When comparing the homospermine homologues 3,7,12,17-tetraazanonadecane, DE(3,4,4), and 3,8,12,17-tetraazanonadecane, DE(4,3,4), the 48 and 96 h IC_{50}'s are the same within experimental error. The compounds' behavior is almost identical to that of DEHSPM. This was also true of 3,8,14,19-tetraazaheneicosane, DE(4,5,4) (**20**), and 3,9,14,20-tetraazadocosane, DE(5,4,5) (**21**) in which the length of DEHSPM is increased by one and two methylenes respectively. It is also notable that going from the shortest diethylated tetraamine, DENSPM, to the longest, DE(5,4,5), the activity increases by a factor of at least 250 at 48 h and by 66 at 96 h. Thus an increase in the size of the methylene backbone proceeding from a (3,3,3), the number of methylenes between nitrogens for DENSPM, through (3,4,3), (4,3,4), (3,4,4), (4,4,4), (4,5,4) to a (5,4,5) array corresponds to a general decrease in the 48 and 96 h IC_{50} values.

Competitive Uptake Determinations in L1210 Cells (K_i Measurement)

The ability of the alkylated norspermines, spermines, homospermines, and homospermine homologues to compete with radiolabeled SPD for uptake was evaluated. The general trend within the norspermine and homospermine series was, that when the terminal alkyl group was larger, the K_i value was larger, Table 5-1. With the norspermines and spermines, the dimethyl, diethyl and dipropyl analogues had very similar K_i values. However, the *iso*-propyl analogue, DIPNSPM, presented a much larger K_i, more than three times higher than that of DPNSPM. The most interesting series was the homospermines. As the terminal alkyl groups went from methyl to *tert*-butyl, a rather impressive change in K_i occurred. The impact of polyamine backbone internal alkylation on the K_i value was substantially different for DENSPM and DESPM. DECroNSPM had

the highest K_i value (97 μM) among all NSPM analogues--more than 6 times than DENSPM. To DECroSPM, the backbone modification seemed to not retard it from competing with SPD for the uptake.

The differences between the ETBHSPM and DTBHSPM K_i values are also notable. The ETBHSPM K_i is slightly higher than that of DEHSPM, while the K_i for DTBHSPM is 40 times higher than the DEHSPM value. This is in keeping with the idea that the polyamine transport apparatus requires only three cationic centers for polyamine recognition and transport (Porter et al. 1985). Thus, one might expect ETBHSPM to bind a little more poorly than DEHSPM as there are two ways DEHSPM could present a nonsterically hindered tricationic array. In DEHSPM an ethyl group, a relatively nonsterically hindered alkyl, is fixed to both ends of the tetraamine. ETBHSPM has an ethyl on one end and a sterically bulky *tert*-butyl group on the other. DTBHSPM has two *tert*-butyl groups, one on each terminus. If the tetracation, or fully protonated tetraamine, binds to the transport apparatus electrostatically, any *tert*-butyl group attached to a cationic center could compromise this interaction by increasing the distance between the charged sites.

Polyamine Pools

Because the IC_{50} curves for the polyamine analogues are sometimes flat near the 50% growth line, it can be difficult to determine the 48 h IC_{50} concentration with great accuracy. Thus, the following guidelines were adopted for studying the impact of the analogues on polyamine pools, Table 5-2. The measurements were made after a 48 h exposure to the analogue, and generally at least two different concentrations of analogue were evaluated in every case except for DEHSPM, which was measured in early work. Except DENSPM, analogues whose IC_{50} concentration exceeded 100 μM at 48 h, the polyamine pools were determined at 100 and 500 μM. The DENSPM was run at 10 and 100 μM. For all other analogues the effect on polyamine pools was evaluated at the 48 h

IC_{50} concentration and at 5 times this number; ETBHSPM was also run at a third concentration, 25 times its 48 h IC_{50}. This was done in hopes of demonstrating that analogues which were not active at 48 h were not effective at depleting polyamine pools in this time frame. Identification of analogues which were not effective at suppressing polyamine pools at 48 h was anticipated.

At 100 μM, the effect of DMNSPM, DENSPM, and MENSPM on polyamine pools was similar, i.e. putrescine was depleted to below detectable limits, and spermidine was reduced to around 5% of controls, while spermine levels were diminished to 36% with DMNSPM and to 11% with MENSPM. Increasing the concentration of the latter two drugs by a factor of 5 had little influence on the pools. The effect of DPNSPM and DIPNSPM on polyamine pools was very similar, and none of them was as effective as the other linear norspermines. With 500 μM DPNSPM or DIPNSPM, spermidine was reduced to 12-19% of control, while putrescine essentially disappeared and spermine remained at 53-56%.

The spermine analogues were slightly more effective than the norspermines at depletion of polyamine pools. In the cases of DMSPM, MESPM and DESPM, at low concentration, putrescine was reduced to below detectable limits, spermidine diminished to under 2% of control, and spermine to under 25%. At 3 μM, DPSPM reduced putrescine to below detection, and spermidine to 18%, while the spermine level remained at 64% of control. At 15 μM DPSPM, spermidine was further reduced to 9% and spermine to 43%. Compared to the analogues in their series, none of the crotyl analgues, DECroNSPD or DECroSPD is very active on polyamine pools.

At their 48 h IC_{50} concentrations, the homospermine analogues, DMHSPM and MEHSPM, were similar to the spermines in their ability to deplete the polyamines. DEHSPM and DIPHSPM were somewhat less effective at suppressing spermine pools. The homospermines with the largest terminal substituents, ETBHSPM and DTBHSPM, were the least effective at depletion of polyamine pools. At 10 μM, ETBHSPM reduced

putrescine to below detectable limits, spermidine to 10% of control, and spermine to 80%. Even at 500 μM, DTBHSPM only reduced putrescine, spermidine, and spermine to 55%, 58%, and 72%, respectively. Finally, the homospermine homologues DE(3,4,4), DE(4,3,4), DE(4,5,4) and DE(5,4,5) all of which demonstrated low 48 h IC_{50} values, did not show substantial depletion of polyamines except at the higher concentration. At this level, their behavior was similar to that of DEHSPM, although they were more effective at depleting spermine.

Although it was not possible to accurately measure the level of the isopropyl, *tert*-butyl and diethylcrotyl analogues in the cells because of method limitations, the concentrations of all of the other analogues could be determined, Table 5-2. The data are not consistent with the idea that the most active compounds are accumulated to the greatest extent.

Impact of Analogues on ODC, AdoMetDC and SSAT

As observed earlier and supported by these studies, there is little correlation between the ODC and AdoMetDC levels and the compound's 48 and 96 h IC_{50} values (Table 5-3). Previous studies suggested that the effect of the polyamine analogues on ODC and AdoMetDC is fairly rapid while analogue-induced upregulation of SSAT activity is somewhat slower (Porter et al.1987a and 1990). For example, DESPM reduction in ODC activity plateaued at 4 h and AdoMetDC at 12 h, while DENSPM induction of SSAT upregulation was maximized at 72 h of exposure to the analogues. On the basis of these studies, we elected to evaluate ODC and AdoMetDC at 4 and 6 h, respectively, and SSAT at 48 h, Table 5-3.

Reduction in ODC activity by DMNSPM, MESPM and DENSPM was rapid. In 4 h, 1 μM DMNSPM, MENSPM, or DENSPM reduced ODC activity to nearly the same extent, to approximately 7% of control. The triamine analogues with larger terminal groups had much less on this enzyme, i.e. DPNSPM only reduced ODC to 79% and DIPNSPM

had no effect at all. The same phenomenon was observed for AdoMetDC. While 1 μM DMNSPM, MENSPM, or DENSPM all reduced the activity to approximately 42% at 6 h, DPNSPM to 70% , and DIPNSPM, again, had no effect on the enzyme. While DENSPM increased SSAT activity by 1500%, MENSPM and PDNSPM increased SSAT by 410-460%, but DMNSPM and DIPNSPM only induced about a 200% upregulation. Except for acetylase activity, similar trends were observed for the spermine analogues. The norspermine analogue with internal side chains, DECroNSPM, had consistently less impact on all of the enzyme levels than its linear homologue DENSPM.

At 1 μM DMSPM, MESPM, or DESPM, ODC activity was reduced to less than 10% of control, while ODC in DPSPM treated cells was only lowered to 52% of controls. DMSPM, MESPM, or DESPM at 1 μM almost paralleled the ability of the corresponding norspermine analogues to suppress AdoMetDC. DPSPM at 1 μM reduced AdoMetDC activity to 72% of that seen in untreated cells. Although none of the spermine analogues was as effective as DENSPM at stimulating SSAT activity, nevertheless, there was a notable increase in activity by three of the four analogues tested. DESPM increased the acetylase activity by 460%, DPSPM by 500%, and DMSPM by 300%. Except for the impact on SSAT, the properties of the corresponding homospermine analogues were very similar to those of the spermines. DMHSPM, MEHSPM, or DEHSPM at 1 μM all reduced ODC to approximately 4% of control. All three of these analogues reduced AdoMetDC to 40%. Once again, DIPHSPM, the diisopropyl compound, had little impact on either ODC or AdoMetDC. ODC was reduced to 90% of control and AdoMetDC to 80%. While ETBHSPM diminished ODC and AdoMetDC to 80% of controls, DTBHSPM had no effect on either enzyme. None of the homospermine analogues, MEHSPM, DEHSPM, DMHSPM, or DIPHSPM at 2 μM approached the level of effect of DENSPM on SSAT activity. MEHSPM had essentially no effect, while DEHSPM, DMHSPM, and DIPHSPM all increased SSAT to about 140%. Of the homospermine homologues, only DE(3,4,4) induced significant upregulation of SSAT activity, that is, 370%. Again,

DECroSPM was less active in reducing ODC and AdoMet, while had similar effect in upregulating SSAT.

A comparison of the homospermine homologues DE(3,4,4) and DE(4,3,4) with DEHSPM itself revealed that moving the three methylene bridges around had little effect on ODC. Interestingly, adding one or two methylene unit to DEHSPM to produce DE(4,5,4) and DE(5,4,5) resulted in a notable decrease in ODC-suppressing activity. ODC was only lowered to 20% of control, while other DE homologues reduced ODC to 5% of control. All three of the analogues were about as effective at reducing AdoMetDC, to about 40%. All of these homospermine homologues had essentially same impact on SSAT as DEHSPM, except DE(3,4,4) which was more active in upregulating SSAT.

The fact that DIPNSPM, DIPHSPM, and ETBHSPM had so little effect on ODC and AdoMetDC under the conditions of the experiment but yet depleted polyamines at 48 h compelled us to look at the effects of these analogues, at least on ODC, after extended exposure and/or at higher concentrations. It is important to recall that all previous experiments were carried out at 1 μM for a 4 h period. This is, of course, in many instances below the 48 h IC_{50} level and in some instances approaches the K_i for the analogue. At 1 μM and 48 h, DIPHSPM reduced ODC to 10% of control. At 1 μM and 48 h, ETBHSPM lowered ODC to 9% of control. At 100 μM DIPNSPM, ODC was lowered to 35% of control, while DTBHSPM had a minimal effect (60%) on ODC under the same conditions.

Biological Evaluation of Triamine Analogues

The triamine analogues are arranged into the following sets of compounds: norspermidines, spermidines, homospermidines, 4,5-triamines, and 5,5-triamines. While the K_i values of norspermidine, spermidine and homospermidine, had been reported long time ago, the Ki values presented in this study were repeated as positive controls, and all of them agree well with the previous ones (Porter & Bergeron 1983).

Antiproliferative Activity - IC_{50} of L1210 cells

As shown in Table 5-4, NSPD is the most active among the NSPD family of analogues with a IC_{50} of 0.9 μM at 48 h and 0.5 μM at 96 h. All of the alkylated analogues give >100 μM IC_{50} values at 48 h. At 96 h the IC_{50}'s range from 3.5 to >100 μM with an order of DMNSPD < DENSPD < MENSPD, MPNSPD and DPNSPD (most to least active). This shows that in this family terminal dialkylation with smaller groups will increase the IC_{50} activity.

At 48 h, SPD and all of its analogues had an IC_{50} over 100 μM. Unlike NSPD, SPD is the least active compounds in its family with IC_{50} values above 100 μM at both 48 and 96h. At 96 h, DMSPD and DESPD showed higher activities than DPSPD. When an ethyl group was removed from either ends of DESPD, a monoalkylated analogue was produced with lower activity than DESPD, in the order of one to two magnitude. However, the monopropyl SPD's have similar activities to DPSPD that all in the range of 20-50 μM at 96 h. It is interesting that monoalkylation (ethyl- and propylation) of SPD at different end results in very different activities. At 96 h, with a IC_{50} of 6 μM, MESPD(N1) was almost 7 times more active than MESPD(N8). The same trend was found among the two monopropyl SPD analogues that MPSPD(N1) was more than twice as active as MPSPD(N8). It seems that alkylation at N^1 position yields a higher activity than alkylation at N^8 (Table 5-4).

Among the HSPD analogs, at 48 h, DEHSPD, as the most active one, gives a IC_{50} of 25 μM, while the others are around or above 100 μM. At 96 h, all of the IC_{50}'s fall into the a range from 0.3 μM ~ 0.9 μM except DPHSPD with a IC_{50} of 6 μM. Compared to the two other families (3,3) and (3,4), the HSPD analogues as a group have higher activity.

As the polyamine chain went up to (4,5) and (5,5), the activity reached the highest in this series for (4,5) analogues and then declined for (5,5) analogues. There is little difference in the dimethyl versus the diethyl in both 4,5- and 5,5-triamine family. The

dipropyl and the parent (non-alkylated) triamines show less activity than the dimethyl or diethyl analogues (Table 5-4). The 96 IC_{50} graph of DE(4,5) was shown in Figure 5-1(a).

Competitive Uptake Determinations in L1210 cells

The ability of the polyamines and their analogues: norspermidines, spermidines, homospermidines, 4,5-triamines and 5,5-triamines to compete with radiolabeled SPD for uptake was evaluated (Table 5-4). The general trend is that the terminal alkylated triamines have higher Ki values than the unalkylated triamines. This demonstrates that the terminal primary amines are essential for polyamine transport, which is consistent with the previous works done by Porter and Bergeron. In the series of spermidines, homospermidines and 4,5-triamines, Ki values increase as the size of the terminal group enlarged. The trend is triamine < DM < ME < DE < DP. This indicated that chemical modification of the primary amines sharply limited the ability of a derivative to compete for the polyamine transport apparatus. Also, aliphatic chain length separating the amines plays a role in determining polyamine uptake ability, even though might be less important than the availability of primary amines.

Polyamine Pools

Because the IC_{50} curves for the polyamine analogues are sometimes flat near the 50% growth line, it can be difficult to determine the 48 h IC_{50} concentration with great accuracy. Thus, the following guidelines were adopted for studying the impact of the analogues on polyamine pools, Table 5-5. The measurements were made after a 48 h exposure to the analogue, and generally at least two different concentrations of analogue were evaluated in every case. Analogues whose IC_{50} concentration exceeded 100 μM at 48 h, the polyamine pools were determined at 100 and 500 μM. For the other analogues, the effect on polyamine pools was evaluated at the 48 h IC_{50} concentration and at 5 times this number.

At 500 μM, the effects of DMNSPD, DENSPD and MPNSPD on polyamine pools were similar (Table 5-5), i.e., PUT was depleted below detectable limits, and spermidine was reduced to 6-15% of controls, while spermine levels were diminished to around 48%. DPNSPD was not as effective as the other norspermidine analogues in polyamine pools, i.e., at 500 μM, PUT was only lowered to 60%, SPD to 71% and no effect on SPM level.

DMSPD and DESPD depleted PUT under detectable limits, SPD to 5%, SPM to 58% and 74% of control, respectively. The monoalkylated SPD analogues MESPD(N^1), MESPD(N^8) and MPSPD(N^1) gave a similar pattern of polyamine pool depletion. At 100 μM, putrescine was depleted below the detectable level, spermidine to 25%, and spermine to 80%, 84% and 90%. MPSPD(N^8) was slightly less active than MPSPD(N^1). At 500 μM, DPSPD reduced PUT below detectable level and SPD around 10% of control. Like DPNSPD, it showed ittle suppression on SPM level even at high concentrations.

Among the homospermidine analogs, the parent triamine, HSPD, showed the highest effect. At 100 μM, PUT was depleted under detectable levels, SPD to 4% and SPM to 32%. For the HSPD analogues, at 500 μM, the level of putrescine was diminished below detectable limits and SPD level below 10% of control. DMHSPD, DEHSPD and MPHSPD had little impact on SPM level. In the case of DPHSPD, the level of SPM seemed to be increased instead of depressed compared to the control.

Similar results were observed in homospermidine homologue (4,5) and (5,5) analogs. At 500 μM, (4,5) and (5,5) depleted both PUT and SPD below detectable level, and SPM to 35% and 20%. Comparatively, the alkylated analogues showed less impact on SPD level (all above from 18%), and barely any impact on SPM. DP(5,5), as the largest analog in this series, only depleted PUT to 51% of control, SPD to 69% of control.

Impact of Analogues on ODC, AdoMetDC and SSAT

In general, the impacts of triamine analogues on the enzyme activities are less effective than the tetraamine analogues. Previous studies suggested that the effect of polyamine analogues, including triamine analogues, on ODC and AdoMetDC is fairly rapid

while analogue-induced upregulation of SSAT activity is somewhat slower. One the basis of these studies, same time frames were selected for the triamine analogues as in the study of tetraamine analogues. The ODC and AdoMetDC were evaluated at 4 h and 6 h, respectively, and SSAT at 48 h, Table 5-6. For both ODC and AdoMet assay, 1 μM of polyamine analogues was used as in the previous study of tetraamines. However, for the SSAT assays, the L1210 cells were treated with 10 μM of triamine analogues, instead of 2 μM used for tetramines.

All of the norspermidine analogues, Table 5-6, except DPNSPD had some effect on ODC activity. The parent triamine norspermidine reduced ODC activity to 11% of control, the corresponding dimethyl, DMNSPD, 17%, the diethyl analogue, DENSPD, to 80% and the dipropyl compound, DPNSPD, had no effect on this enzyme. Monoethylnorspermidine, MENSPD, was more active than the corresponding dialkyl analogue, DENSPD, reduction to 42 *vs* 80% of control, as was the monopropyl, MPNSPD, relative to its dipropyl counterpart DPNSPD, reduction to 33 *vs* 100% of control. Although there is no strictly proportional relationship between K_i and ODC, a rough trend can be found in which a compound with the lower K_i value tends to show higher impact in reducing ODC activity during a 4 h treatment period. The reduction of AdoMetDC activity by the norspermidines was about 50% except in the case of DPNSPD, which had essentially no impact on the enzymes. At 10 μM, while DENSPD increased SSAT activity by 780%, MPNSPD and MENSPD increased SSAT by 470% and 390%, respectively, but DMNSPD and DPNSPD only induced about a 250% upregulation. The parent triamine NSPD had the least activity on inducing SSAT activity, only to 150% of control.

The spermidine analogues are more effective at reducing ODC activity than norspermidines. At 1 μM, the parent triamine spermidine reduced ODC to 16% of control while the alkylated analogues except for dipropylspermidine, DPSPD, diminished ODC activity to between 10-30% of control. Again the monoalkyl analogues are more effective than the corresponding dialkyl compounds. Both N^1 and N^8 monoethyl spermidine

MESPD(N^1), MESPD(N^8), are more active than DESPD reducing ODC to 10 and 17% *vs* 30% of control respectively. This property of the monoalkylated analogue is even further accentuated with the propylated spermidines MPSPD(N^1), MPSPD(N^8) *vs* DPSPD lowering ODC to 18 and 14% *vs* 75% of control. Again when comparing dialkylated compounds the larger the alkyl substituent the less active the analogue. Compared to the corresponding norspermidines, the spermidine analogues were less effective at reducing AdoMet activity. The spermidine analogues except for DPSPD reduced AdoMet to around 60% of control activity. DPSPD has little impact on the enzyme. Again as with ODC, the monoalkylated spermidine compounds were generally more active than the dialkylated compounds. At 10 μM, DESPD, MPSPD(N^1) and DPSPD significantly stimulated SSAT activity, 1370%, 1200% and 1030% of control, respectively. The other spermdine analogues except SPD also induced the acetylase activity by several folds, i.e. MPSPD(N^8) increased the SSAT activity by 510%, MESPD(N^1) by 430%, MESPD(N^8) by 400%, and DMSPD by 270%. In contrast, the parent spermidine only induced the acetylase activity to 150% of control.

The homospermidines were very similar to the spermidines at reducing ODC actvity. Also, in consistent with the other norspermidine and spermidine results, the triamines with the larger substituent, propyl, were least effective and the monoalkyl compounds were more active than the dipropyl triamines. The homospermidine analogues were not effective at AdoMetDC under the condition of assay. Although none of the homospermidine analogues was as effective as DESPD at stimulating SSAT activity, nevertheless, there was a notable increase in activity by all of the analogues tested, including parent homospermidine. Homospermidine increased the acetylase activity by 430%, DMHSPD by 510%, DEHSPD by 630%, MPHSPD by 560% and DPHSPD by 410%.

Interestingly, adding a methylene unit to alkylhomospermidine to produce dialkyl (4,5) and dialkyl (5,5) compound, the ODC-suppressing and the SSAT-inducing capacity

and substantially decreased while the AdoMet properties were similar to those of the homopsermidines.

Acute and Chronic Toxicity of Triamines

In early studies of polyamine toxicity in laboratory animals, triamines were found less toxic than tetraamines. For example, spermidine was approximately one-twentieth as nephrotoxic as spermine (Tabor & Rosenthal 1956), and the acute LD_{50} of spermidine was at least three times higher than spermine (Shaw 1972).

In the current study, the acute toxicity of six triamines and the chronic toxicity of two triamines were measured (Table 5-7). The value of all polyamine LD_{50}s are shown in both mg/kg and mmole/kg for comparison.

For acute toxicities, the polyamine analogues were administered as a single i.p. injection to groups of five or six CD-1 female mice (average 35 g) at each dose. The animals were scored two hours after administration of drug. All animals that survived at two hours were observed for 10 days to assess late onset of toxicity from the single acute dose. From Table 5-7, it is clear that the acute LD_{50}s for triamine analogues are approximately twice the acute LD_{50}s for the corresponding tetraamine analogues.

In the chronic toxicity regimen, mice were administered the polyamine analogue in three doses per day (t.i.d.) for six days for a total of eighteen injections per animal. Appetite, weight and overall physical appearance were monitored on a daily basis. Surviving animals were observed for 10 days after the final dose for lethalities. At least three test groups of six animals each, representing at least three different dose levels, were evaluated for each analogue tested. These three dose levels, estimated on the basis of the acute toxicity results, were carefully chosen so that at least two test groups presented with lethalities, one with a high fraction (> 0.50) of lethalities.

The most active triamine DE(4,5) against L1210 cells in vitro and the spermidine analogue DE(3,4) demonstrated much less toxicity in mice than the corresponding

tetraamine DE(4,5,4), DE(5,4,5) and DE(3,4,3) respectively. In our early study of tetraamines, a preliminary investigation suggested a direct ratio relationship between IC_{50} value and the chronic LD_{50} values. However, in the triamine systems, the 96 h IC_{50} values of DE(4,5) suggested that this triamine should be about 5 times less toxic than corresponding tetraamine analogue, DE(4,5,4) and six times less toxic than DE(5,4,5) (Table 5-1 and Table 5-4), but in fact they are about eleven fold less toxic than the DE(4,5,4) and about fourteen fold less toxic than DE-(5,4,5) (Table 5-7). A similar difference is also observed in the chronic toxicity of DE(3,4). A comparison of the tetraamine and triamine by 96 IC_{50}, suggests the triamine should be approximately 4 times less toxic but it is at least six times less toxic than DE(3,4,3). Thus the potential widening of the therapeutic window renders the triamine analogues promising antineoplastics with low toxicity and encourages us to pursue further animal studies.

The effect of DE(4,5) on GI tract was investigate at the dosage of 150 µmole/kg. In contrast to the severe inhibition of DEHSPM in gastric emptying at the same dosage, no inhibition effect was observed in DE(4,5) treated animals.

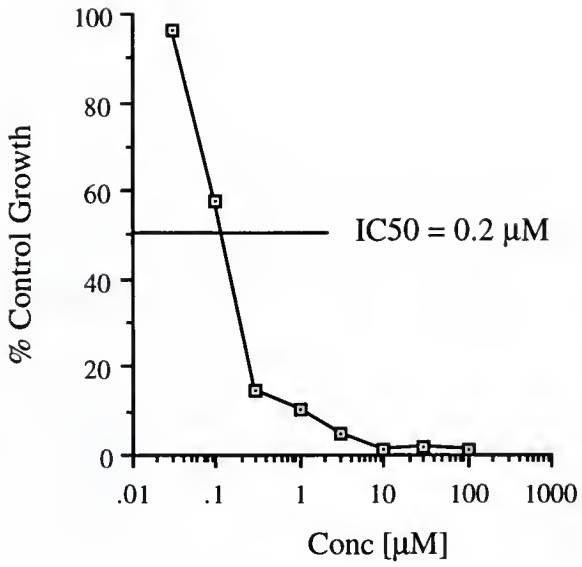
Conservation of Charge

In an earlier study we noted that there was a conservation of charge with regards to the total polyamine cationic picoequivalence in the cell (Bergeron 1989). For example if after 24 h of exposure, each of the equivalent concentration associated with charge on the amines is added together, the numbers are fairly constant. In order to maintain this balance of charge, the cell processes the natural polyamines e.g. exports them as it incorporates the analogues. For example, each picoequivalence of putrescine associated with two picoequivalence of cationic charge, each picoequivalence of spermidine or its alkylated analogues with three and each picoequivalence of spermine with four. The maintenance of total cellular charge holds for all of the triamines examined, Table 5-8, except the 5,5-triamines. The implication is that the cell will not incorporate analogue beyond a point

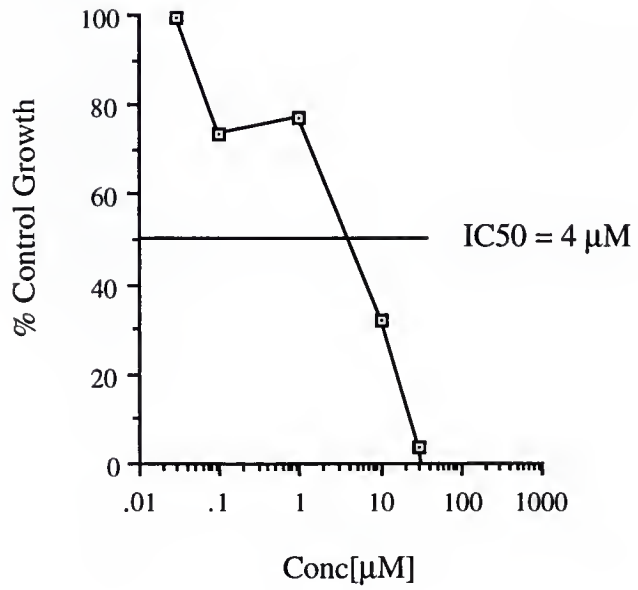
where the charge balance is disrupted at which time cell death may occur. In the case of the tetraamines, the conservation of charge behavior seems to hold for 24 h but erodes after a period of time.

Antiproliferative Activity - IC_{50} of MALME-3M Cells

The 96h IC_{50} of human melanoma MALME-3M was shown in Figure 5-1(b). Compared to the tetraamine analogues which were found active at the level of 0.1 to 10 μM, the IC_{50} value of DE(4,5), 4 μM, demonstrated that DE(4,5) is very potent in suppressing the proliferation of human cancer cells.



(a)



(b)

Figure 5-1: 96 h cell growth vs. DE(4,5) concentration.
(a) Mice leukemia L1210 cells, doubling time = 12 h.
(b) Human melanoma MALME-3M cells, doubling time = 48-72 h.

Table 5-1: Tetraamine analogues structures, abbreviations, L1210 growth inhibition, and transport.

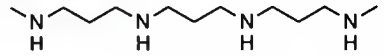
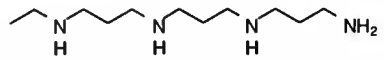
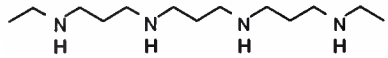
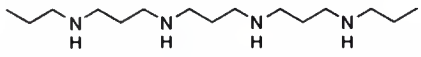
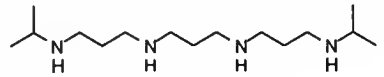
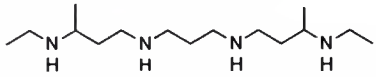
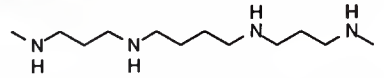
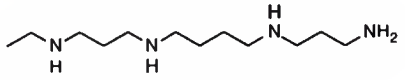
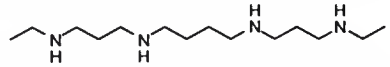
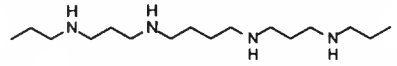
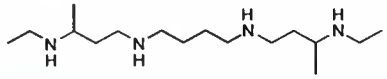
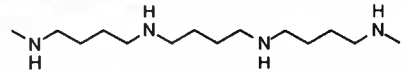
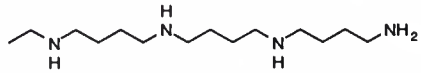
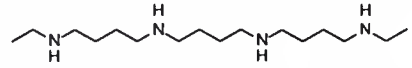
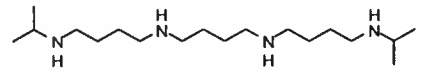
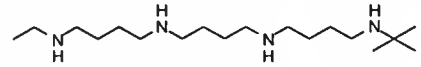
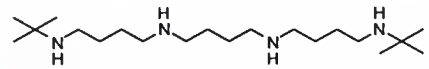
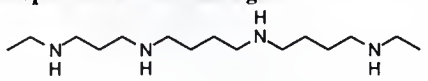
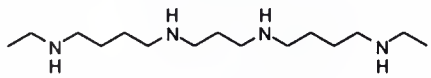
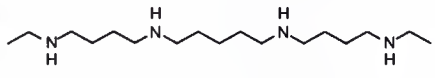
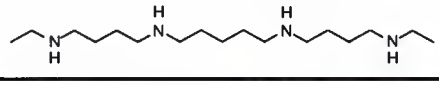
	Structure	Abbreviation	IC_{50} (μM) 48 h	96 h	K_i (μM)
Norspermines					
1		DMNSPM	>100	2.5	5.6
2		MENSPM	>100	2.5	7.7
3		DENSPM	>100	2	17
4		DPNSPM	>100	18	11
5		DIPNSPM	>100	1.2	40
6		DECroNSPM	>100	0.8	97
Spermines					
7		DMSPM	>100	0.75	1.1
8		MESPM	99	0.33	1.7
9		DESPM	30	0.18	1.6
10		DPSPM	3	0.2	2.3
11		DECroSPM	2.5	0.6	1.9
Homospermines					
12		DMHSPM	>100	0.32	0.97
13		MEHSPM	14	0.17	1.1
14		DEHSPM	0.2	0.07	1.4
15		DIPHSPM	20	0.06	8.1
16		ETBHSPM	0.4	0.03	3
17		DTBHSPM	>100	3	56

Table 5-1---continuted

	Structure	Abbreviation	IC_{50} (μM) 48 h	96 h	K_i (μM)
Homospermine homologues					
18		DE(3,4,4)	0.3	0.08	8
19		DE(4,3,4)	1	0.18	4
20		DE(4,5,4)	0.3	0.035	6.0
21		DE(5,4,5)	0.4	0.03	16

K_i values and IC_{50} concentrations at 48 and 96 h. K_i determinations were made by following analogue inhibition of spermidine transport.

Table 5-2: Impact of tetraamine analogues on polyamine pools.

	Compd	Conc.(μM)	Put	Spd	Spm	Analogue
		Norspermines				
1	DMNSPM	100	0	5	36	2.14
		500	0	3	27	1.84
2	MENSPM	100	0	5	11	1.69
		500	0	2	11	1.72
3	DENSPM	10	30	14	31	1.59
		100	0	6	30	2.44
4	DPNSPM	100	61	35	77	0.89
		500	1	19	56	1.28
5	DIPNSPM	100	10	26	64	-
		500	0	12	53	-
6	DECroNSPM	100	0	24	66	-
		500	0	14	54	-
		Spermines				
7	DMSPM	100	0	0	21	1.26
		500	0	0	24	1.24
8	MESPM	100	0	1	21	1.24
		500	0	1	19	1.23
9	DESPM	30	0	0	12	0.40
		150	0	0	14	1.13
10	DPSPM	3	0	18	64	1.12
		15	0	9	43	1.09
11	DECroSPM	3	0	10	56	-
		15	0	5	38	-

Table 5-2--continuted

	Compd	Conc.(μM)	Put	Spd	Spm	Analogue
			Homospermines			
12	DMHSPM	100	0	0	30	1.49
		500	0	0	27	1.03
13	MEHSPM	14	0	0	26	3.22
		70	0	0	26	2.99
14	DEHSPM	10	0	0	61	2.94
15	DIPHSPM	20	0	17	83	-
		100	0	7	67	-
16	ETBHSPM	0.4	133	96	75	-
		2.0	78	68	63	-
		10	0	10	77	-
17	DTBHSPM	100	83	85	94	-
		500	55	58	72	-
			Homospermine homologues			
18	DE(3,4,4)	0.3	40	47	65	0.27
		1.5	0	0	18	0.64
19	DE(4,3,4)	1	81	41	52	0.76
		5	0	0	27	1.50
20	DE(4,5,4)	0.3	44	61	70	0.26
		1.5	0	5	31	0.72
21	DE(5,4,5)	0.15	37	55	88	0.34
		0.75	0	10	58	1.48

Putrescine (Put), spermidine (Spd), and spermine (Spm) levels after 48 h of treatment are given as % polyamine found in untreated controls. Analogue amount is expressed as nmole/10^6 cells.

Table 5-3: Effect of tetraamine analogues on ornithine decarboxylase (ODC), S-Adenosyl-methionine decarboxylase (AdoMetDC), and Spermidine/Spermine N^1-Acetyltransferase (SSAT) in L1210 cells.

	Compd	ODC	AdoMetDC	SSAT
	Norspermines			
1	DMNSPM	6	49	200
2	MENSPM	5	33	410
3	DENSPM	10	42	1500
4	DPNSPM	79	70	460
5	DIPNSPM	100 (35)b	100	180
6	DECroNSPM	56	97	410
	Spermines			
7	DMSPM	3	40	300
8	MESPM	10	27	150
9	DESPM	3	28	460
10	DPSPM	52	72	500
11	DECroSPM	24	56	490
	Homospermines			
12	DMHSPM	4	45	140
13	MEHSPM	3	41	110
14	DEHSPM	7 (1)	41	140
15	DIPHSPM	90 (10)	78	130
16	ETBHSPM	79 (9)	81	-
17	DTBHSPM	100 (60)b	100	-
	Homospermine homologues			
18	DE(3,4,4)	5	37	370
19	DE(4,3,4)	5	28	120
20	DE(4,5,4)	20	39	120
21	DE(5,4,5)	19	54	190

Enzyme activity is expressed as percent of untreated control for ODC (1 μM at 4 h (or 48 h)), AdoMetDC (1 μM at 6 h) and SSAT (2 μM at 48 h). [b]100 μM at 48 h.

Table 5-4: Triamine analogue structures, abbreviations, L1210 growth inhibition, and transport.

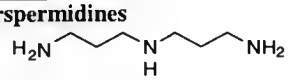
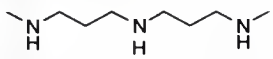
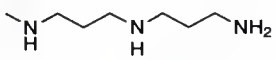
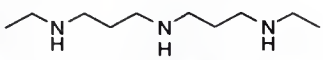
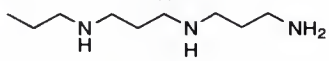
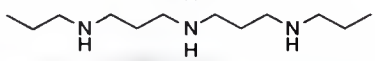
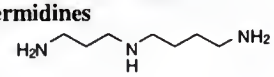
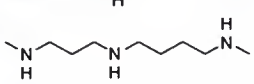
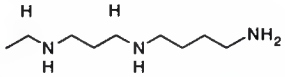
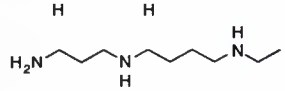
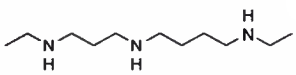
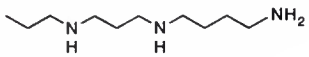
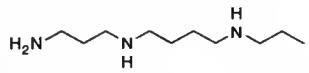
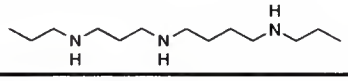
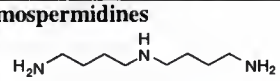
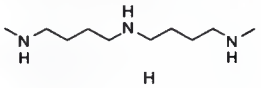
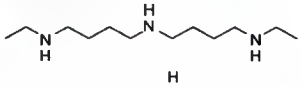
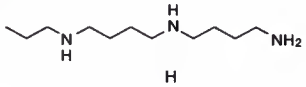
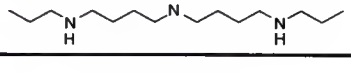
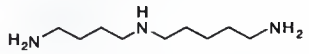
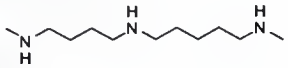
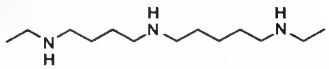
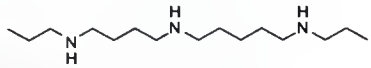
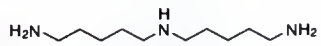
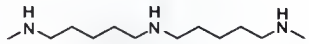
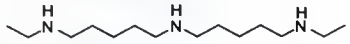
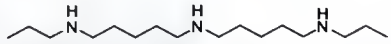
	Structure	Abbreviation	IC_{50} (μM) 48 h	96 h	K_i (μM)
Norspermidines					
22		NSPD	0.9	0.5	7.2
23		DMNSPD	>100	3.5-6.0	60
24		MENSPD	>100	>100	34
25		DENSPD	>100	10	250
26		MPNSPD	>100	~100	33
27		DPNSPD	>100	60	125
Spermidines					
28		SPD	>100	>100	2.2
29		DMSPD	>100	1.5-1.8	5.1
30		MESPD(N1)	>100	3.0-5.0	8.6
31		MESPD(N8)	>100	40	7
32		DESPD	~100	0.6-0.8	19.3
33		MPSPD(N1)	>100	20-35	3.0
34		MPSPD(N8)	>100	50-60	8.5
35		DPSPD	>100	30-35	25.6
Homospermidines					
36		HSPD	>100	1.7-4.0	3.4
37		DMHSPD	>100	0.9	5.5
38		DEHSPD	18-25	0.3-0.4	19
39		MPHSPD	100	0.5-0.7	5.0
40		DPHSPD	>100	6.0	67

Table 5-4--continuted

Structure	Abbreviation	IC_{50} (μM) 48 h	96 h	K_i (μM)
4,5-Triamines				
41 	4,5-Triamine	>100	0.15-0.20	1.4
42 	DM(4,5)	2.0	0.11-0.12	21
43 	DE(4,5)	3.0-6.0	0.19-0.20	64
44 	DP(4,5)	~100	1.0-1.4	75
5.5-Triamines				
45 	5,5-Triamine	~100	0.3-0.5	13.8
46 	DM(5,5)	15	0.4	133
47 	DE(5,5)	10-12.5	0.65-0.70	174
48 	DP(5,5)	>100	6.0	87

K_i values and IC_{50} concentrations at 48 and 96 h. K_i determinations were made by following analogue inhibition of spermidine transport. The IC_{50} values represented the results from at least two measurements, and in each measurement triplicate samples were used.

Table 5-5: Impact of triamine analogues on polyamine pools.

	Compd	Conc.(μM)	Put	Spd	Spm	Analogue
			Norspermidines			
22	NSPD	0.9	38	44	113	1.09
		4.5	0	12	83	2.14
23	DMNSPD	100	0	9	58	5.00
		500	0	6	48	5.51
25	DENSPD	100	0	17	74	3.67
		500	0	7	47	3.77
26	MPNSPD	100	0	29	56	3.07
		500	0	15	49	4.78
27	DPNSPD	100	70	76	96	0.49
		500	68	71	102	1.24
			Spermidines			
28	SPD	100	0	117	118	—
		500	0	145	108	—
29	DMSPD	100	0	5	58	4.96
		500	0	0	54	4.89
30	MESPD(N1)	100	0	25	80	4.20
		500	0	14	53	4.73
31	MESPD(N8)	100	0	26	84	4.41
		500	0	15	61	4.96
32	DESPD	100	0	5	74	4.61
		500	0	0	55	4.20
33	MPSPD(N1)	100	0	25	90	3.97
		500	0	15	72	4.95
34	MPSPD(N8)	100	0	33	103	3.52
		500	0	16	95	5.16
35	DPSPD	100	6	35	135	3.26
		500	0	12	99	3.69

Table 5-5--continuted

	Compd	Conc.(μM)	Put	Spd	Spm	Analogue
		Homospermidines				
36	HSPD	100	0	4	32	3.58
		500	0	2	21	4.44
37	DMHSPD	100	0	3	106	5.51
		500	0	0	106	5.85
38	DEHSPD	25	0	6	114	4.61
		125	0	3	97	4.69
39	MPHSPD	100	0	2	82	5.16
		500	0	0	66	5.86
40	DPHSPD	100	0	19	144	3.12
		500	0	7	111	3.68
		Homospermidine homologues				
41	4,5	100	0	1	53	3.02
		500	0	0	35	3.20
42	DM(4,5)	2	0	33	112	2.79
		10	0	18	111	5.36
43	DE(4,5)	3	0	47	99	1.20
		15	0	18	98	3.40
44	DP(4,5)	100	0	40	119	1.40
		500	0	31	121	2.42
45	5,5	100	0	0	33	2.58
		500	0	0	20	2.50
46	DM(5,5)	15	0	33	115	2.56
		75	0	20	101	3.61
47	DE(5,5)	15	0	55	97	1.09
		75	0	23	73	1.59
48	DP(5,5)	100	59	73	97	0.86
		500	51	69	103	1.33

Putrescine (Put), spermidine (Spd), and spermine (Spm) levels after 48 h of treatment are given as % polyamine found in untreated controls. Analogue amount is expressed as nmole/10^6 cells.

Table 5-6: Effect of triamine analogues on ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), and spermidine/spermine N^1-acetyltransferase (SSAT) in L1210 cells.

.

	Compd	ODC	AdoMetDC	SSAT
	Norspermidines			
22	NSPD	11	62	150
23	DMNSPD	17	41	250
24	MENSPD	42	58	390
25	DENSPD	80	45	780
26	MPNSPD	33	38	470
27	DPNSPD	100	99	220
	Spermidines			
28	SPD	16	43	160
29	DMSPD	22	68	270
30	MESPD(N1)	10	58	430
31	MESPD(N8)	17	54	400
32	DESPD	30	68	1370
33	MPSPD(N1)	18	56	1200
34	MPSPD(N8)	14	64	510
35	DPSPD	75	107	1030
	Homospermidines			
36	HSPD	11	54	430
37	DMHSPD	20	86	510
38	DEHSPD	47	90	630
39	MPHSPD	20	59	560
40	DPHSPD	86	123	410

Table 5-6--continuted

	Compd	ODC	AdoMetDC	SSAT
		4,5-triamines		
41	4,5-triamine	19	57	410
42	DM(4,5)	56	71	130
43	DE(4,5)	100	70	120
44	DP(4,5)	83	86	80
		5,5-triamines		
45	5,5-triamine	16	88	90
46	DM(5,5)	105	97	90
47	DE(5,5)	100	109	90
48	DP(5,5)	73	123	90

Enzyme activity is expressed as percent of untreated control for ODC (1 μM at 4 h (or 48 h)), AdoMetDC (1 μM at 6 h) and SSAT (10 μM at 48 h). The results are expressed in the means of triplet measurements.

Table 5-7: Comparison of the acute and chronic toxicity of tetraamine and triamine analogues on mice.

TETRAAMINES			TRIAMINES		
Compd	Acute[a] LD_{50} mg/kg (mmole/kg)	Chronic[b] LD_{50} mg/kg-day (mmole/kg-day)	Compd	Acute LD_{50} mg/kg (mmole/kg)	Chronic LD_{50} mg/kg-day (mmole/kg-day)
DE-[3,4,3]	340 (0.842)	87 (0.215)	DE-[3,4]	> 650[d] (> 3.22)	426 (1.37)
DE-[4,5,4]	285 (0.638)	48 (0.104)	DE-[4,5]	555 (1.64)	375 (1.11)
DE-[5,4,5]	195 (0.424)	36 (0.078)	DE-[5,5]	500 (1.42)	nd

All of the polyamine analogues were administrated in the form of hydrochloride salts. [a]Single-dose ip. [b]Multiple-Dose ip (tid x 6 days). [c]At a single dose of 250 mg/kg, no death within the initial 2 h, but all six animals were expired within seven days. [d]15 mg/kg (t.i.d x 3 day), 4/5 died on day 6 and 5/5 died on day 7. [e]At a single dose of 600 mg/kg, no death within the initial 2 h, but 5/5 died within seven days. nd = non determined.

Table 5-8: Summation of intracellular levels of analogues and polyamines analyzed for amine equivalence after exposure to polyamine analogues.

	Polyamine Analogues	Nanoequivalents of Amine /10^6 cells	Average ± Standard Deviation
Control Cell		13.21	
2	DMNSPD	18.40	
4	DENSPD	13.70	
5	MPNSPD	17.71	
6	DPNSPD	15.01	16.21 ± 2.22
8	DMSPD	16.09	
9	MESPD(N1)	16.99	
10	MESPD(N8)	17.99	
11	DESPD	14.05	
12	MPSPD(N1)	18.25	
13	MPSPD(N8)	19.59	
14	DPSPD	15.33	16.90 ± 1.89
16	DMHSPD	20.34	
17	DEHSPD	16.92	
18	MPHSPD	20.55	
19	DPHSPD	15.99	18.45 ± 2.34
21	DM(4,5)	20.81	
22	DE(4,5)	14.59	
23	DP(4,5)	15.15	16.85 ± 3.44
24	DM(5,5)	15.5	
25	DE(5,5)	9.01	
26	DP(5,5)	13.91	12.81 ± 3.38
All Analogues		Mean	16.47 ± 2.08

The L1210 cells were treated with polyamine analogues at 500 μM, except DEHSPD (125 μM), DM(4,5) (10 μM), DE(4,5) (15 μM), DM(5,5) (75 μM) and DE(5,5) (75 μM), for 48 h. Levels of amine equivalence for every analogue treated cells are averages from analysis of triplicate cell samples. Values are obtained by multiplying the number of moles of spermine by four, spermidine by three, putrescine by two and analogue by three. The typical control values in nmol/million L1210 cells are PUT = 0.260 ± 0.059, SPD = 3.354 ± 0.361, SPM = 0.658 ± 0.119.

CHAPTER 6
METABOLISM OF POLYAMINE ANALOGUES

Introduction

Polyamine oxidase (PAO) is an important catabolic enzyme evolved in the interconversion pathway of polyamines (Seiler 1995). It is responsible for the oxidation of spermidine and spermine in rat liver (Hölttä 1977). Spermidine was shown to be degraded to putrescine and 3-aminopropion-aldehyd. Spermine was found to divide into to spermine and 3-aminopropionaldehyde. Later, Bolkenius and Seiler (1980) demonstrated that N^1-acetylspermidine, N^1-acetylspermine and N^1,N^{12}-acetylspermidine were better substrates for PAO than the non-acetylated polyamines. In enzyme reactions, PAO attacks the secondary nitrogen linked to a propylacetylamide and splits it to form a shorter polyamine, an aldehyde (3-acetamidopropanal) and hygrogen peroxide. It has been shown that PAO from mammalian cells will not metabolize N^8-acetyl spermidine in contrast to N^1-acetylspermidine, i.e. PAO requires an acetylated aminopropyl group (Seiler 1995).

Polyamine oxidase is a flavinprotein, containing a tightly bound flavin adenine dinucleotide (FAD) as a prosthetic group. Molecular oxygen is the usual electron acceptor (Hölttä 1977). The enzyme activity has been reported to be unaffected by the addition of NAD, NADH, or NADPH, although NADP was shown to be inhibitory at mM concentrations (Hölttä 1977). Unlike the regulatory enzymes involved in the polyamine metabolism, the activity of polyamine oxidase is constantly high and not significantly effected by various growth situations (Hölttä 1977).

In an experiment focused on the impact of dipropylnorspermidine, DPNSPD, on polyamine pools a rather substantial unexpected peak appeared in the chromatogram of treated cells. This peak was later shown to correspond to monopropylnorspermidine, MPNSPD (Table 6-1, Figure 6-1). The levels of MPNSPD in the cells after a 48 h exposure to DPNSPD was about 50% of the parent compound. While the tetraamines clearly sustained mono and di-dealkylation in animals previous studies with the alkylated tetraamines (Bergeron et al. 1995a and 1996a). e.g. diethylhomospermine, DEHSPM or diethylspermine, DESPM, in L1210 cells revealed either little or no N-dealkylation under the conditions of experiments. The observation of *N*-depropylation of DPNSPD compelled us to look more closely at the metabolism of the other polyamine analogues in L1210 cells. A careful inspection of chromatograms from cells treated with DMNSPD, DMSPD, DMHSPD, DM(4,5) and DM(5,5), revealed no dealkylation, Table 6-1. Among the diethylated triamine analogues, DENSPD is the only one clearly showed a metabolite, monoethylnorspermidine, MENSPD at 0.245 nmol/10^6 cells (about 4% of the parent compound). DESPD and DEHSPD might show some metabolites, but hardly above detectable levels. Neither DE(4,5) or DE(5,5) gives detectable level of metabolite. The level of monodealkylation seen in cells treated with DPNSPD, 50% of intracellular parent drug, was significant higher than its dimethyl and diethyl analogues. The importance of the tirmaine backbone in N-depropylation was selected for investigation.

Of the five different dipropyl triamines which were evaluated DPNSPD, DPSPD, DPHSPD, DP(4,5) and DP(5,5), all but DE(5,5) showed significant N-depropylation. The HPLC chromatogram of the L1210 cells incubated with various analogues at 100 μM for 48 h are shown in Figure 6-2 to Figure 6-7. The metabolic pathway is shown schematically in Figure 6-1, and the distribution of metabolites in Table 6-1. As seen in Table 6-1, *N*-depropylation is in general more active than deethylation. The dipropyl triamine with the shortest backbone DPNSPD seemed most sensitive to metabolism with MPNSPD representing 50% of the total drug. With DPSPD both monoalkylated products

N^1-monopropylspermidine [MPSPD(N^1)] and N^8-monopropylspermidine [MPSPD(N^8)] were detected, each at a level of 0.130 nmole/million cells, 4% of parent triamine. When cells were exposed to DPHSPD, only a single product was observed at 12% of DPHSPD, MPHSPD. Interestingly, cells converted DP(4,5) to a single compound at 0.546 nmole/10^6 cells, 39% of the total cellular DP(4,5). When DP(5,5) was evaluated no metabolic products were found, suggesting that the aminobutyl end of DP(4,5) system was selectively dealkylated.

In an attempt to assess whether or not the culture media was responsible for dealkylation, we explored the effects of various media components on the analogues were explored (Table 6-2). Fetal bovine serum (FBS) for example, is well-known to contain amine oxidases (Morgan 1989). Indeed, the 1 mM aminoguanidine, a well known inhibitor of bovine serum amine oxidase (Gahl & Pitot 1978) in our standard L1210 cell culture media, did not totally eliminate such FBS-related amine oxidase activity. A small amount of the DPNSPD was metabolized to MPNSPD extracellularly. But the comparatively low extracellular concentration of MPNSPD (~ 3 μM) and its relatively poor affinity (K_i = 33 μM) for the polyamine transport apparatus, argue against the extracellular medium as a major source of the intracellular MPNSPD (264 μM). This conclusion is further supported by experiments which partially or totally eliminate the extracellular metabolism. When FBS was replaced with either NuSerum, a semi-synthetic substitute, or albumin a high level of intracellular metabolite (50% of parent analogue) was still observed. When DPNSPD was exposed to FBS in the presence of bathophenanthroline disulfonic acid, no MPNSPD was observed. However, when cells were grown in RPMI, FBS and bathophenanthroline disulfonic acid, DPNSPD was converted very effectively to MPNSPD as 50% of DPNSPD. This is in keeping with the idea that the dealkylation indeed takes place in the cells.

Among all of the reported amine oxidases, polyamine oxidase (PAO) is the only one which attacks at a secondary amine center, three methylene carbons internal to the

primary amine termini of spermine for example. It is believed that polyamine oxidase may recognize such a similarity between its native substrate and N-propyl analogues, and catalyze N-depropylation. In order to verify this hypothesis, the metabolism of DPNSPD using partial purified PAO in cell free system was studied.

Experimental

Chemicals and Reagents

Putrescine, N^1-acetyl putrescine, N^1-acetyl spermidine, N^8-acetyl spermidine, N^1-acetyl spermine, DL-dithiothreitol, homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid), polyethylene glycol were from Sigma Chemical Co. (St. Louis, MO). MPNSPD, DPNSPD, N^1-propyl spermidine [MPSPD(N^1)], N^8-propyl spermidine [MPSPD(N^8)], DPSPD, MPHSPD, DPHSPD, DP(4,5) and DP(5,5) were synthesized in this study. Boric acid, sodium tetraborate, EDTA were from Fisher Scientific Co. Horseradish peroxidase and fetal bovine serum and bovine albumin was obtained from Sigma. NuSerum IV was purchased from Collaborative Biomedical Products (Bedford, MA). Bathophenanthrolinedisulforic acid was from Aldrich Chemical Co. Serva Hydroxyapatite (cellulose ion exchanger) was from Gallard-Schlesinger Chem. MFG. Corp. (Garden City, N.Y.).

Study of Polyamine Metabolism in Cell Culture

Standard complete media and cell culture condition

Murine L1210 leukemia cells were maintained in logarithmic growth as a suspension culture in RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS), 2% HEPES-MOPOS buffer, and 1 mM aminoguanidine at 37 °C in a water-jacket 5% CO_2 incubator.

Standard method for study of polyamine metabolism

While in logarithmic growth, cells were treated with the polyamine derivatives at 100 μM and 500 μM, and incubated for 48 h at 37 °C. At the end of the treatment period,

cell suspensions were sampled, washed twice in cold fresh RPMI-1640 medium (2 x 10 mL) and centrifuged. The supernatant was carefully removed and the cells resuspended in 0.6 M perchloric acid (1 x 10^7 cells/mL). The cells were then freeze-fractured by four successive freeze-thawing using liquid nitrogen. When necessary, cell lysates were stored at -20 °C until analysis by HPLC analysis.

HPLC analysis

A 100-µL aliquot of polyamines in 0.6 N HCl was reacted with 400 µL dansyl chloride (5 mg/mL in acetone), sodium carbonate (0.12 g) and an appropriate internal standard (*e.g.*, 30 µL of 1.25 x 10^{-4} M 1,6-diaminohexane). After vortexing, the reaction mixture was heated at 70 °C for 20 min. and again vortexed and cooled to room temperature (20 min.). An aqueous proline solution (100 µL of 250 mg/mL) was added to quench the excess dansyl chloride and allowed to react at room temperature for 10 min. The dansylated polyamines were transferred using 25% aqueous CH_3CN (3 x 500 µL) to a solid phase extraction column which had been prepared by successive rinses of MeOH and aqueous CH_3CN. The column was aspirated, rinsed with 25% aqueous CH_3CN (2 x 2.5 mL), the dansylated polyamines were eluted with 4 mL MeOH (2 x 2 mL) and analyzed by the Rainin Instrument Company HPLC system, which incorporated control and data acquisition by a microcomputer using Rainin Dynamax software and hardware. Automatic sample injection was performed via a 20-µL fixed sample loop on the Rheodyne injection valve of a Gilson autosampler/diluter. Fluorescence detection by a Hewlett-Packard HP1046A (ex = 228 nm; em = 495 nm) or McPherson (ex = 360 nm; em = 530 nm) programmable detector was employed. A linear gradient program from 65% CH_3CN in water to 93% MeOH in water for 28 min and a Symmetry C-18 column 4.6 mm x 250 mm, 5-µm (Waters) were used for separation .

Study of polyamine metabolism by media components I, in absence of L1210 cells

Similar to the above assay, but with the absence of L1210 cells, the mixture of complete media containing FBS, and polyamine analogues was incubated and the samples were then treated with 0.6 M perchloric acid (1:1, v/v).

Study of polyamine metabolism by media compoents II, in absence of L1210 cells

NuSerum IV is a semi-synthetic bovine fetal serum replacement, and contains only 25% fetal bovine serum. The composition of the complete media is same as standard complete media except 10% NuSerum IV replaces FBS.

Study of metabolism by L1210 cells in serum free system

Bovine albumin (1.5%) was used in place of bovine serum. The maximum incubation time was 4 h before a significant loss of cell viability (< 70%). The cells were then harvested and prepared for HPLC analysis.

Study of Metabolism in Cell-Free System

Partial purification of polyamine oxidase

Liver was obtained from Male Sprague-Dawley rats, weighing 350-400 g. The polyamine oxidase was purified as described by Bolkenius and Seiler (1981) and Hölttä (1983). The hydroxyapatite-purified enzyme was used in kinetic studies. The purification procedure is shown in a flow chart (Figure 6-8) and the enzyme activity was purified by a factor of 20. The specific activity of the purified enzyme and the overall yield are comparable with the results found in literature. The preparation did not contain significant diamine oxidase or monoamine oxidase activities, as was demonstrated by the absence of hydrogen peroxide formation with putrescine, N^1-acetylputrescine and N^8-acetylspermidine as substrates. In the case of N^8-acetylspermidine, HPLC also verified the lack of detectable putrescine generated in the system.

Detection of PAO activity by HPLC

A standard incubation mixture contained 200 μM of substrate and 300 μg of enzyme in a total volume of 1.5 mL of borate buffer pH 9.0. The reaction mixture was incubated at 37 °C with agitation. At each time interval, a 200 μL of reaction mixture was sampled and the reaction was stopped by addition of 40 μL of 1 N HCl. The samples were frozen at -20 °C until analysis by HPLC.

Detection of PAO activity by fluorescence assay

PAO activities were determined fluorometrically by the method measuring the H_2O_2 produced by PAO by using horseradish peroxidase-coupled conversion of homovanillic acid to a highly fluorescent compound (Snyder & Hendley 1968). The standard incubation mixture contained 200 μM polyamine substrate, 1.0 mM homovanillic acid, 40 μg horseradish peroxidase and an appropriate amount of PAO enzyme preparation in a total volume of 0.5 mL 0.07 M potassium phosphate buffer pH 7.8. The mixture was incubated at 37 °C in air with agitation. The enzyme reaction was stopped by cooling in an ice-water bath, and then 2.0 mL 0.1 N NaOH was added to each sample (Suzuki et al. 1984). Fluorescence intensity was measured immediately using a Perkin-Elmer Fluorescence Spectrophotometer LS-3B. Fluorescence was activated at 323 nm and emission was measured at 426 nm. Blanks containing enzyme protein, but no polyamine substrate were incubated as usual and the substrate added after addition of 0.1 N NaOH solution. For the measurement of enzyme kinetics, the amount of protein and incubation times were adjusted to provide a linear reaction rate.

A stock solution of quinine sulfate (0.3 mg/mL) in 0.1 N HCl was used routinely as a reference standard in correction of fluorescence readings. The stock solution was diluted 1000 times before reading, and this fluorescence reading was recorded. The readings of the samples were corrected according to the following formula and expressed in Fluorescence Unit.

Fluorescence Unit = (sample reading - blank reading) / (quinine sulfate reading)

Calibration of standard hydrogen peroxide

A calibration curve was determined by measuring the Fluorescence Unit of a group of samples containing increasing concentrations of freshly prepared standard hydrogen peroxide (instead of polyamine substrate) after 5 min incubation at 37 °C. The exact concentration of the stock H_2O_2 solution was determined by titration with $KMnO_4$ (Welcher). The resultant Fluorescence Units were used to calculate equivalent nanomoles of substrate oxidized per minute.

Protein determination

Protein was determined by measuring the ultraviolet absorption at $\lambda = 280$ nm (Dawson et al. 1986).

Data Processing

Velocity measurements were carried out in a period from 30 min up to 2 h. Initial velocities were obtained by using the program in Cricket Graph. K_m values were determined by double reciprocal plots of initial velocities as a function of substrate concentrations. The slope and intercept were determined graphically. The maximum velocity was determined graphically by Hanes plot ([S]/Vvs. 1/[S]).

Results

The activity of partially purified polyamine oxidase was studied using either N^1-acetylspermine or DPNSPD as substrate. In each study, the generation of SPD or MPNSPD was detected by HPLC (Figure 6-9). During a typical 12 hour incubation, the formation of SPD from N^1-acetylspermine remained linear with respect to time, indicating that N^1-acetylspermine was a very good substrate of the enzyme preparation. The formation of MPNSPD from DPNSPD showed that the enzyme preparation was also substantially active in catalyzing N-depropylation of DPNSPD (Figure 6-8). However, in contrast to the formation of spermidine from N^1-acetylspermine, the rate of depropylation began to decline after the first 1 h of incubation (Figure 6-8). It seems that a product of

DPNSPD depropylation, either MPNSPD or propanal, may be acting as a feedback inhibitor of PAO, although the nature of this inhibition remains to be clarified.

The substrate specificity of polyamine oxidase was also studied, using the peroxidase-coupled fluorescence reaction for the establishment of the kinetic parameters. The results of the enzyme kinetic measurements are summarized in Table 6-3. The *N*-depropylation was investigated at pH 7.8 because this is a system similar to the biological system (Suzuki et al. 1981). As shown in Table 6-3, N^1-acetylspermine and N^1-acetylspermidine were very good substrates for polyamine oxidase and their V_{max} (Bolkenius & Seiler 1981) and the K_m values (Bitonti et al. 1990) are in agreement with the values previously reported for using partial or highly purified PAO. DPNSPD is also good substrate of this enzyme purification with a V_{max} of 1.0 nmole/min/mg protein, about one third of that of N^1-acetylspermine.

The PAO enzyme preparation was also studied for its activity towards several other polyamines which have been shown to be poor substrates for PAO, but good substrates for other amine oxidases. N^1-Acetylputrescine and N^8-acetylspermidine were good substrates for monoamine oxidase (flavin-dependent amine oxidase), and putrescine and N^8-acetylspermidine were good substrates for the copper-dependent amine oxidases. However, none of these polyamines indicated metabolization by the PAO enzyme preparation (Table 6-3). This suggest that the partial purified PAO does not contain significant activity of other amine oxidases.

The effects of different enzyme cofactors were also investigated. The enzyme reaction was not stimulated either by NADPH (2 mM) or FAD (2 μM). These observations suggest that the *N*-depropylation of DPNSPD was not due to a P_{450}-N-dealkylation enzyme nor did the enzyme have loosely bound FAD.

Discussion

N^1-Acetylspermine and N^1-acetylspermidine are the natural substrates for polyamine oxidase and are much better substrates than their unacetylated counterparts, spermine and spermidine. Structurally an acetylated aminopropyl group is required for the polyamine oxidase catalyzed degradation. Comparing the terminal propyl group of DPNSPD with the propylamide terminal of N^1-acetylspermine, it is interesting to find that both of the groups had a neutral hydrophobic three carbon methylene chain. This similarity may allow DPNSPD to be recognized by the enzyme active site.

Hydrogen peroxide and mono- and di-*N*-depropylated metabolites were all detected as reaction products of polyamine oxidase catalyzed *N*-depropylation. The enzyme reaction also produced a dinitrophenoylhydrazine positive product suggesting the formation of an aldehyde or ketone (Bolkenius and Seiler 1989). In this study, the amount of *N*-dealkylated triamine(s) formed is in a 1:1 stoichiometric relationship with the amount of hydrogen peroxide as shown below:

$$\text{n-Propyl-NH(CH}_2)_m\text{NH(CH}_2)_n\text{NH-n-Propyl} \rightarrow \text{n-Propyl-NH(CH}_2)_m\text{NH(CH}_2)_n\text{NH}_2 + H_2O_2 + CH_3CH_2CHO$$

$$\text{n-Propyl-NH(CH}_2)_m\text{NH(CH}_2)_n\text{NH}_2 \rightarrow \text{NH}_2\text{(CH}_2)_m\text{NH(CH}_2)_n\text{NH}_2 + H_2O_2 + CH_3CH_2CHO$$

The proposed enzymatic reaction above, is illustrated in Figure 6-11 (B), for comparision to the deaminopropylation of N^1-Acetylspermine [Figure 6-11 (A)].

Most of the documented dealkyltion were observed in the terminal benzylated polyamine analogues. The N-dealkylation of polyamine analogues by PAO was first observed in the study of *N*-benzyl and *N,N'*-bis(benzyl) derivatives of the homologous aliphatic diamines (Bolkenius &Seiler 1989) (Figure 6-12). Another investigation by Bitonti et al. (1990) demonstrated a series of *N,N'*-bis(benzyl)tetraamine analogues were

substrates for highly purified rat liver polyamine oxidase. The tetraamines shared a common structure as shown in Figure 6-12. In the same study, benzylamine was also found to be a substrate for PAO.

A structure-activity comparison of both the natural and synthetic polyamine analogues as substrates of PAO revealed some very interesting features of PAO-catalyzed N-dealkylation. As shown in Figure 6-12, when the polyamine analogues are lined up by the nitrogen atom which attacked by PAO, it is clear that there are some structural requirements for a polyamine analogue as a good substrate of PAO.

First, a hydrophobic and bulky terminal alkyl group serves better as a substrate than a charged or less bulky terminal group, i.e. N^1-acetylspermine is a much better substrate than spermine and the dimethyl triamines are distinguished from their dipropyl analogues by not giving any detectable metabolite(s). The propyl groups of the dipropyl triamines might resemble the acetyl group of N^1-acetylspermine, providing these triamines with a high affinity towards PAO.

Second, a proper length of carbon chain between the external and internal nitrogens is required. Among our propyl triamines, the pattern of the metabolism of five different dipropyl triamines were investigated (Figure 6-1). The symmetric molecules, DPNSPD and DPHSPD, each gave one metabolite, MPNSPD and MPHSPD respectively. In contrast, the asymmetric DPSPD presented two metabolites MPSPD(N^1) and MPSPD(N^8) each in identical quantities. Interestingly, the asymmetric triamine DP(4,5) only produced a single metabolite peak, and DP(5,5) did not give any metabolite. It is clear that the presence of either a propyl or a butyl methylene chain between the terminal nitrogen and the internal nitrogen is essential for N-depropylation. In accordance with the above study is the observation that for the benzylated diamines, the analogues of diaminopropane and putrescine were much better substrates than the diaminopentane analogues (Bolkenius & Seiler 1989). This is further demonstrated by the dibenzylated tetraamine series as all of these analogues had a three or four methylene chain between

the external and internal nitrogens (Bitonti et al. 1990). It seems that further structure modification beyond the second nitrogen will not impact the property of analogue as a PAO substrate.

Thus, in general, a polyamine backbone with two positively-charged amino groups separated by three or four methylene carbons is required to serve as a substrate for PAO. This is consistant with the reported PAO metabolism of the mono- and di-benzylated diamines, while in this case, an analogue with five carbon backbone is a substrate, although a poor one.

There is considerable evidence showing that polyamine analogues are *N*-alkylated in vivo in rodents, dogs and humans. *N*-dealkylation of DENSPM, DESPM and DEHSPM have been clearly demonstrated in this laboratory (Bergeron et al. 1995a and 1996a). Prakash (1990) also reported that the livers from mice treated with *N,N'*-bis(3-(ethylamino)propyl)1,7-heptanediamine (DEPH), a homologue of DESPM, contained two apparent metabolites which were suspected to be mono- and di-*N*-dealkylation products. Pretreatments with a reported specific inhibitor of PAO inhibitor, BBP (*N,N'*-bis-2,3-butadienylputrescine), considerably decreased the amount of the two metabolites and at the same time, potentiated the antitumor activity of DEPH.

Our in vitro study using purified polyamine oxidase clearly demonstrate that DPNSPD is a good substrate of PAO and DENSPD is a substrate of PAO, albeit a relatively poor one. However DMNSPD is not a substrate of PAO or is, at best, an extremely poor one. This substrate specificity of purified rat liver PAO towards *N*-propyl, *N*-ethyl and *N*-methyl polyamine analogues paralleled the metabolite pattern observed in L1210 cells. These results suggest that the polyamine oxidase may play an important role in the *N*-dealkylation of bisalkyl polyamines in L1210 cells, and perhaps in other tissues in vivo. Furthermore, the structure-activity relationship of PAO substrates provides us valuable information in design of prodrugs either as specific enzyme inhibitors or as antiproliferative reagents with suitable half lives.

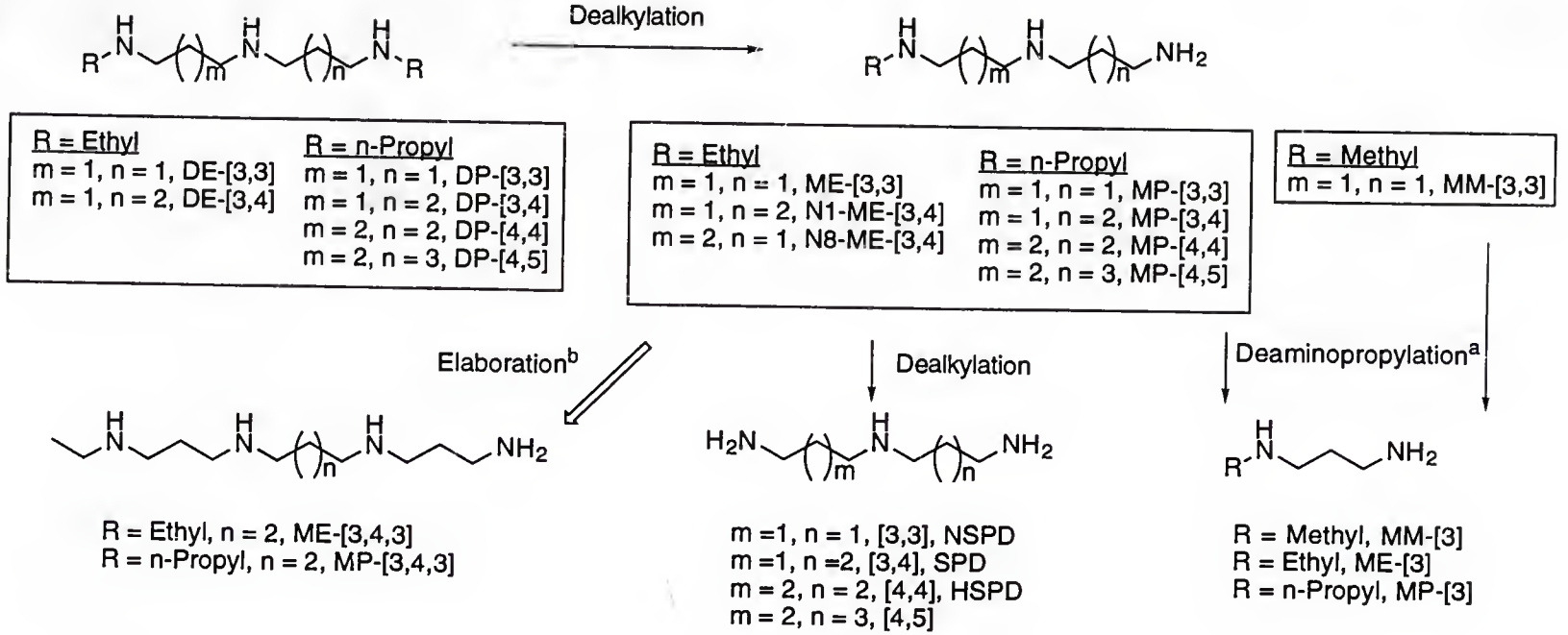
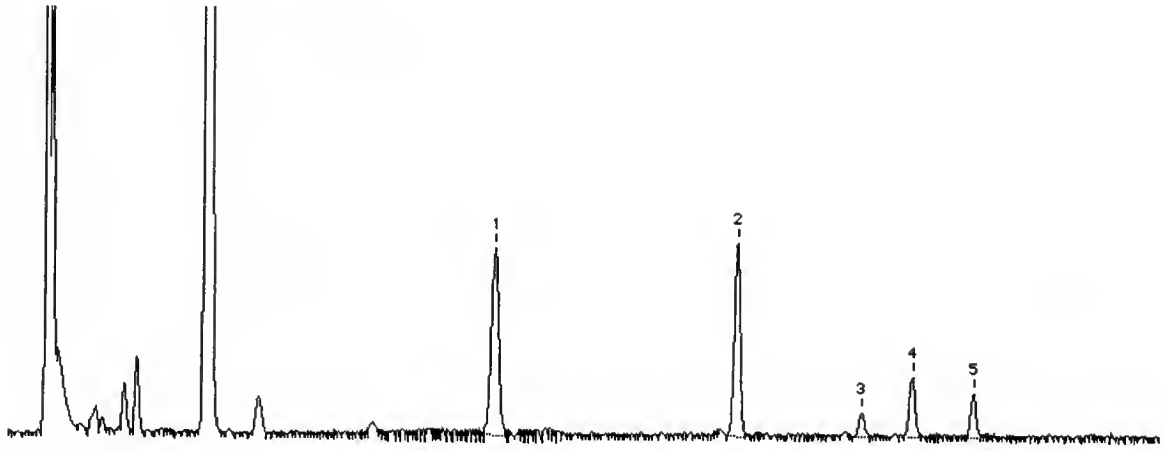


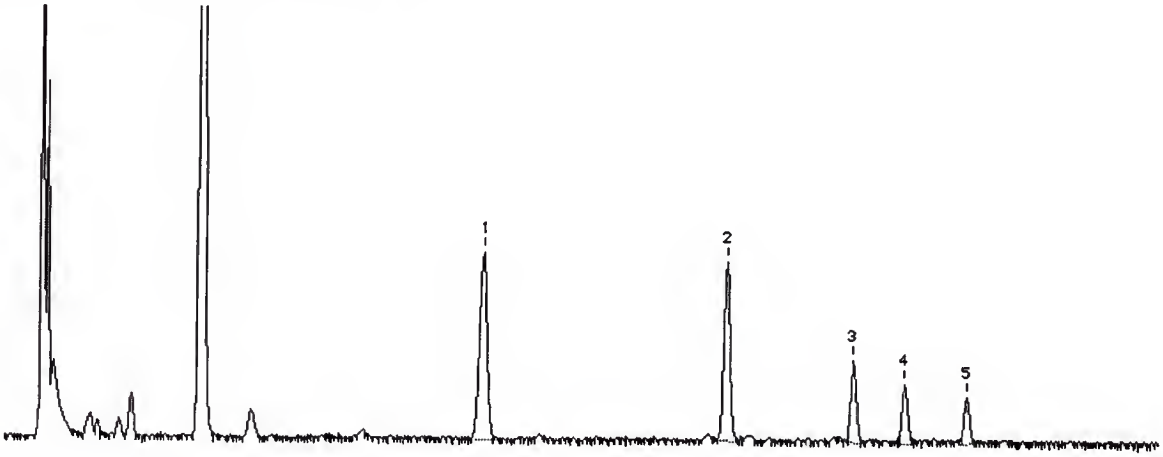
Figure 6-1: Structures of alkylated polyamines involved in dealkylation biotransformation.
[a]The deaminopropylation happens to the primary amino end. [b]Elaboration, the enlongation of polyamine backbone, by addition of an amino propyl group. In this series, it only happened to the aminobutyl end of N^1-alkylated SPD. e.g. spermine synthase catalyzed reaction.

(a) DPNSPD 100 μM



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	14.705	4322	54259	44.901	DHX
2	22.033	4484	42095	34.835	SPD
3	25.798	542	4164	3.445	?
4	27.333	1407	12560	10.393	SPM
5	29.151	998	7763	6.424	DPNSPD
Total Area			120841	99.998	

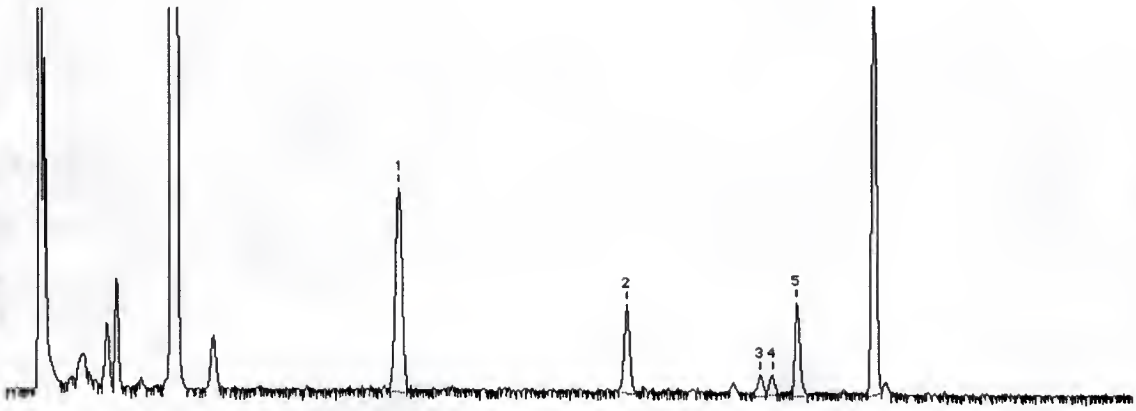
(b) + MPNSPD 849 pmloe/100 mL



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	14.506	4387	55712	42.151	DHX
2	21.858	4188	39325	29.752	SPD
3	25.668	1907	16372	12.386	MPNSPD
4	27.211	1393	11885	8.992	SPM
5	29.061	1079	8878	6.717	DPNSPD
Total Area			132172	99.998	

Figure 6-2: HPLC chromatogram of metabolism study of DPNSPD.
(a) Sample was taken from L1210 cells treated with DPNSPD (100 μM).
(b) sample of (a) spiked with MPNSPD (849 pmole/100 μL)).

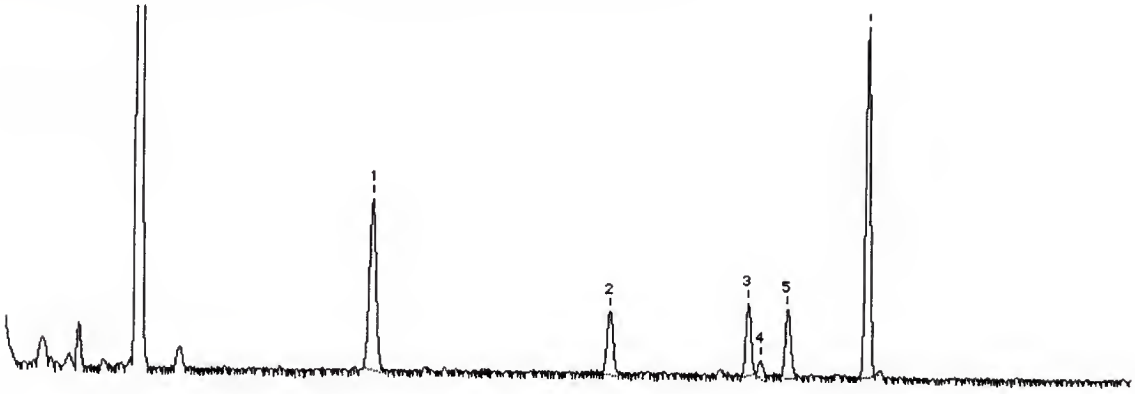
(a) DPSPD 100 μM



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	13.190	3864	50191	32.551	DHX
2	20.893	1673	16231	10.526	SPD
3	25.391	400	3665	2.376	?
4	25.788	393	2895	1.877	?
5	26.613	1777	15469	10.032	SPM
6	29.203	7970	65737	42.634	DPSPD
Total Area			154188	99.996	

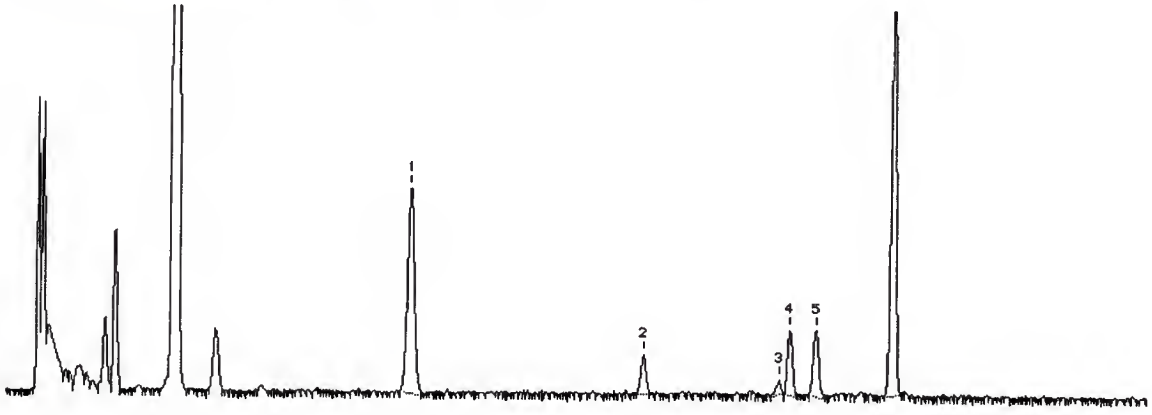
Figure 6-3: HPLC chromatogram of the metabolism study of DPSPD.
(a) Sample was taken from L1210 cells treated with DPSPD (100 μM).

(b) DPSPD 100 μM + 782 pmoles/100 μL MPSPD(N^1)



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	13.210	4287	51303	30.235	DHX
2	20.913	1590	16423	9.678	SPD
3	25.430	1803	15056	8.873	MPSPD(N1)
4	25.846	424	3435	2.024	?
5	26.701	1708	15998	9.428	SPM
6	29.283	8756	67465	39.760	DPSPD
Total Area			169680	99.998	

(c) DPSPD 500 μM + 600 pmoles/100 μL MPSPD(N^8)



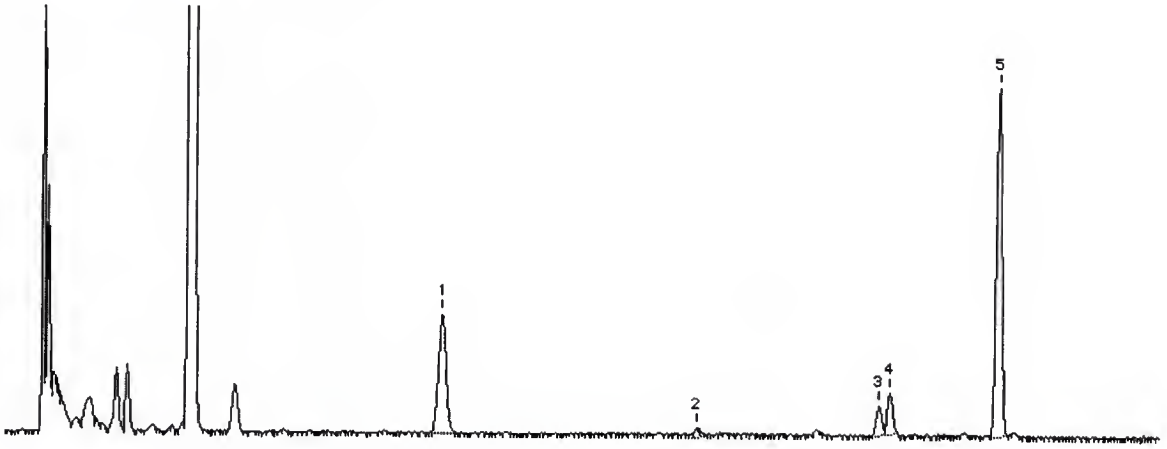
Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	13.465	4211	53319	34.600	DHX
2	21.211	797	7000	4.542	SPD
3	25.681	292	1146	0.743	MPSPD(N1)
4	26.043	1296	10821	7.022	MPSPDN8)
5	26.898	1351	12232	7.937	SPM
6	29.450	8299	69580	45.153	DPSPD
Total Area			154098	99.997	

Figure 6-3--continued.

(b) Sample of (a) spiked with MPSPD(N^1) (782 pmole/100 μL).

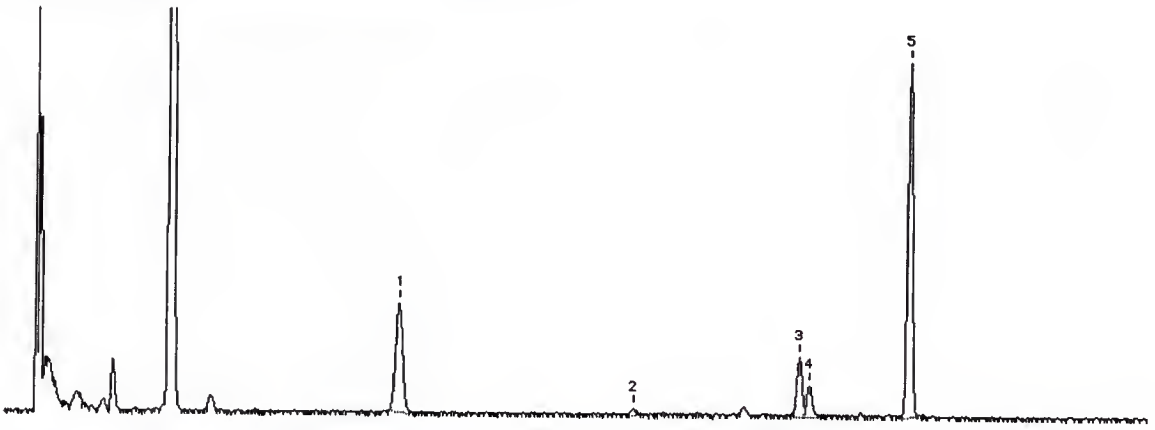
(c) Sample of 500 μM DPSPD treated L1210 cells spiked with MPSPD(N^8) (600 pmole/100 μL).

(a) DPHSPD 500 μM



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	13.188	3934	50103	29.276	DHX
2	20.906	303	3499	2.044	SPD
3	26.371	972	8082	4.722	?
4	26.693	1321	10396	6.074	SPM
5	29.943	11563	99057	57.881	DPHSPD
Total Area			171137	99.997	

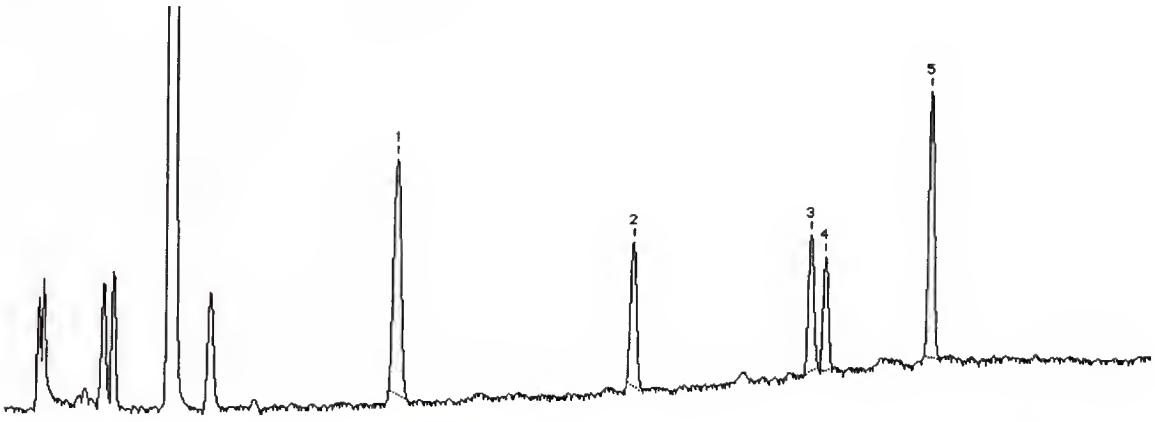
(b) DPHSPD + 600 pmole/100 μL MPHSPD



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	13.108	3982	52732	27.758	DHX
2	20.873	240	1491	0.784	SPD
3	26.356	2201	19351	10.186	MPHSPD
4	26.698	1111	10744	5.655	SPM
5	29.951	12962	105647	55.613	DPHSPD
Total Area			189965	99.996	

Figure 6-4: HPLC chromatogram of the metabolism study of DPHSPD.
(a) Sample was taken from L1210 cells treated with DPHSPD (500 μM).
(b) Sample of (a) spiked with MPHSPD (600 pmloe/100 μL).

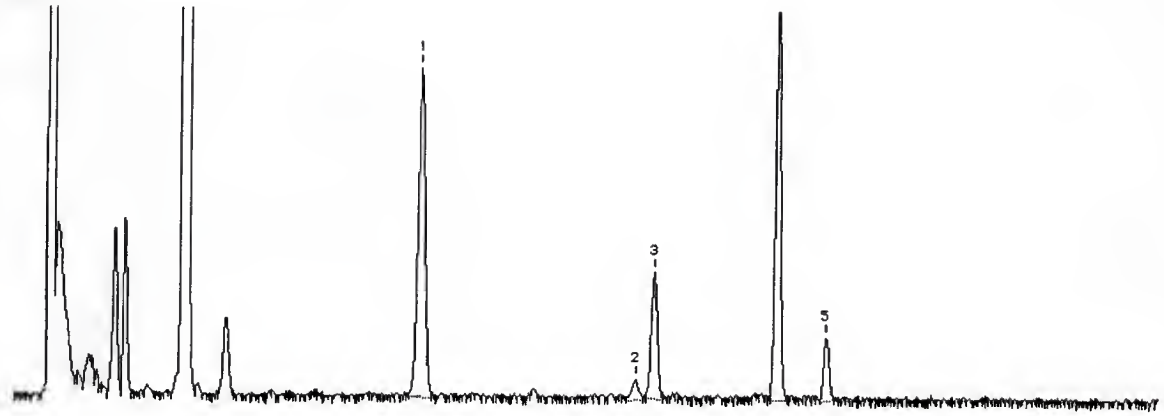
DP(4,5) 100 μM



Peak No	Time	Height(μV)	Area	Area%	Peak Name
1	12.985	2127	30184	33.715	DHX
2	20.818	1294	14490	16.185	SPD
3	26.681	1236	12205	13.633	SPM
4	27.163	1026	9706	10.841	?
5	30.623	2417	22940	25.624	DP(4,5)
Total Area			89525	99.998	

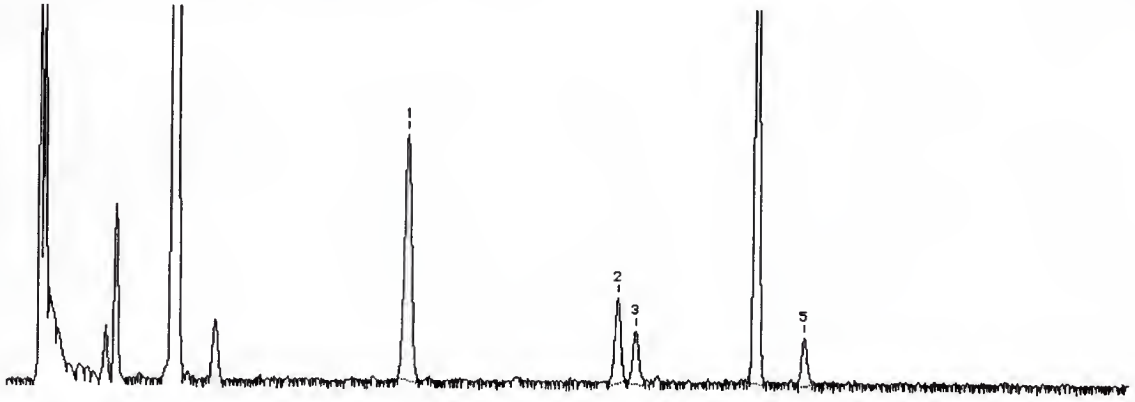
Figure 6-5: HPLC chromatogram of the metabolism study of DP(4,5). Sample was taken from L1210 cells treated with DP(4,5) (100 μM).

(a) MPNSPD 100 µM



Peak No	Time	Height(µV)	Area	Area%	Peak Name	Conc.
1	13.468	5685	74280	43.296	DHX	3750.000
2	20.601	356	3697	2.154	?	
3	21.225	2207	22708	13.236	SPD	931.947
4	25.276	6944	61876	36.066	MPNSPD	3052.882
5	26.888	1076	9001	5.246	SPM	349.637
Total Area			171562	99.998		

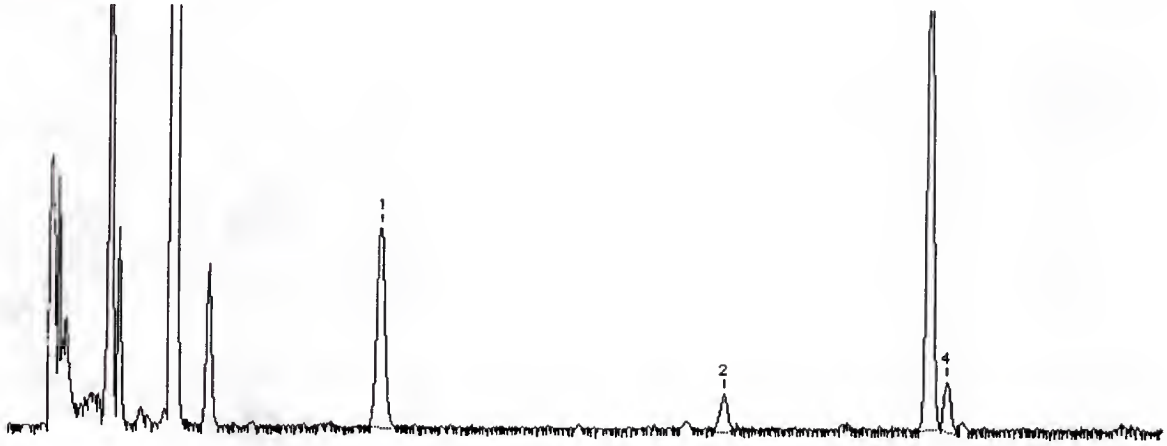
(b) MPNSPD + 900 pmole/100 µL NSPD



Peak No	Time	Height(µV)	Area	Area%	Peak Name	Conc.
1	13.550	4405	59334	33.973	DHX	3750.000
2	20.633	1514	16202	9.276	NSPD	
3	21.271	950	9570	5.479	SPD	491.691
4	25.300	8937	82130	47.026	MPNSPD	5072.918
5	26.933	864	7411	4.243	SPM	360.389
Total Area			174647	99.997		

Figure 6-6: HPLC chromatogram of the metabolism study of MPNSPD.
(a) Sample was taken from L1210 cells treated with MPNSPD (100 µM).
(b) Sample (a) spiked with NSPD (900 pmole/100 µL).

MPHSPD 100 μM



Peak No	Time	Height(μV)	Area	Area%	Peak Name	Conc.
1	11.276	3483	48978	30.820	DHX	3750.000
2	21.671	664	8126	5.113	HSPD	
3	27.861	8660	92487	58.198	MPHSPD	5212.640
4	28.376	850	9325	5.867	SPM	531.944
Total Area			158916	99.998		

Figure 6-7: HPLC chromatogram of the metabolism study of MPHSPD.
The sample was taken from L1210 cells treated with MPHSPD (100 μM).

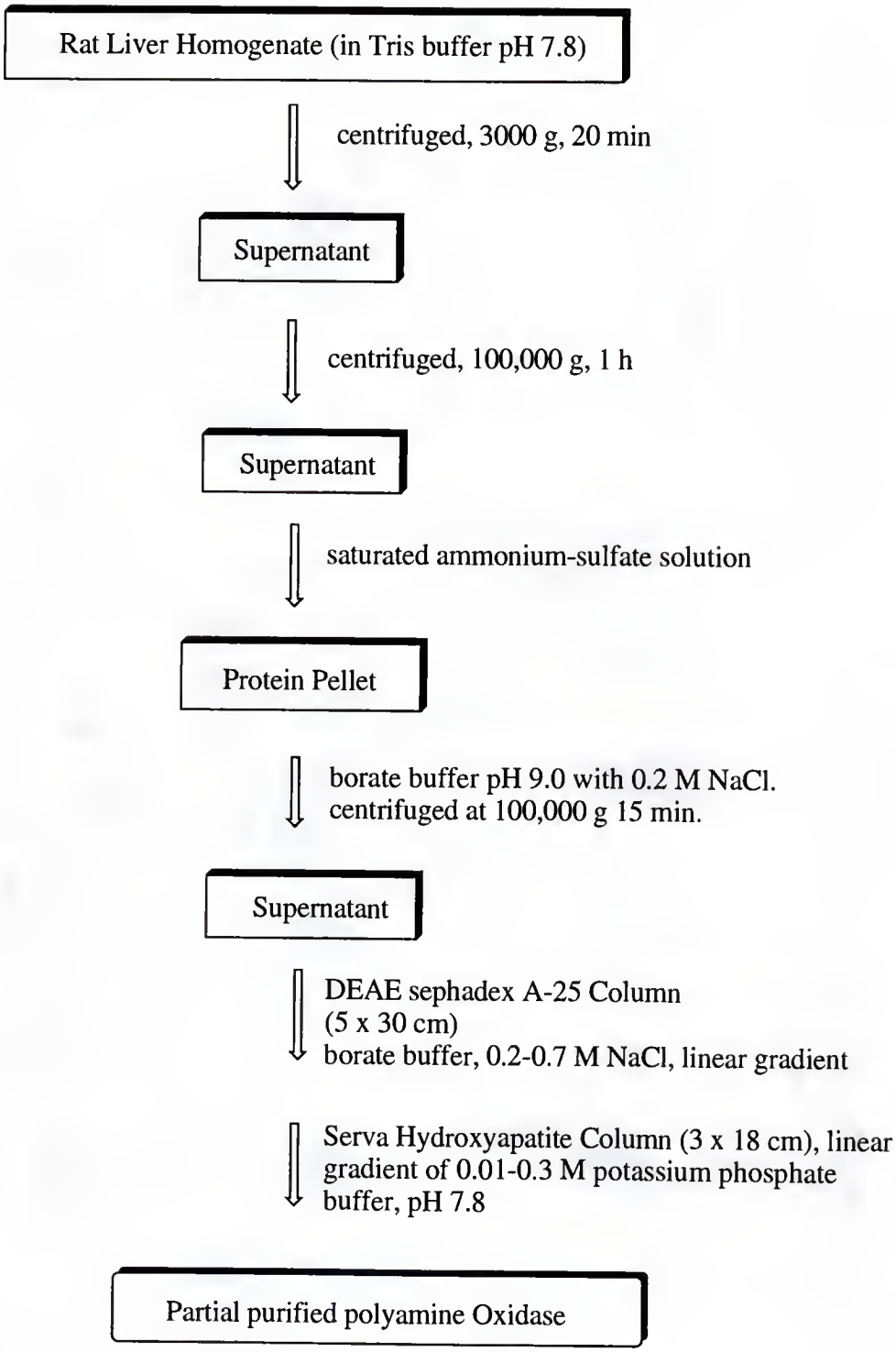


Figure 6-8: Purification of rat liver polyamine oxidase.

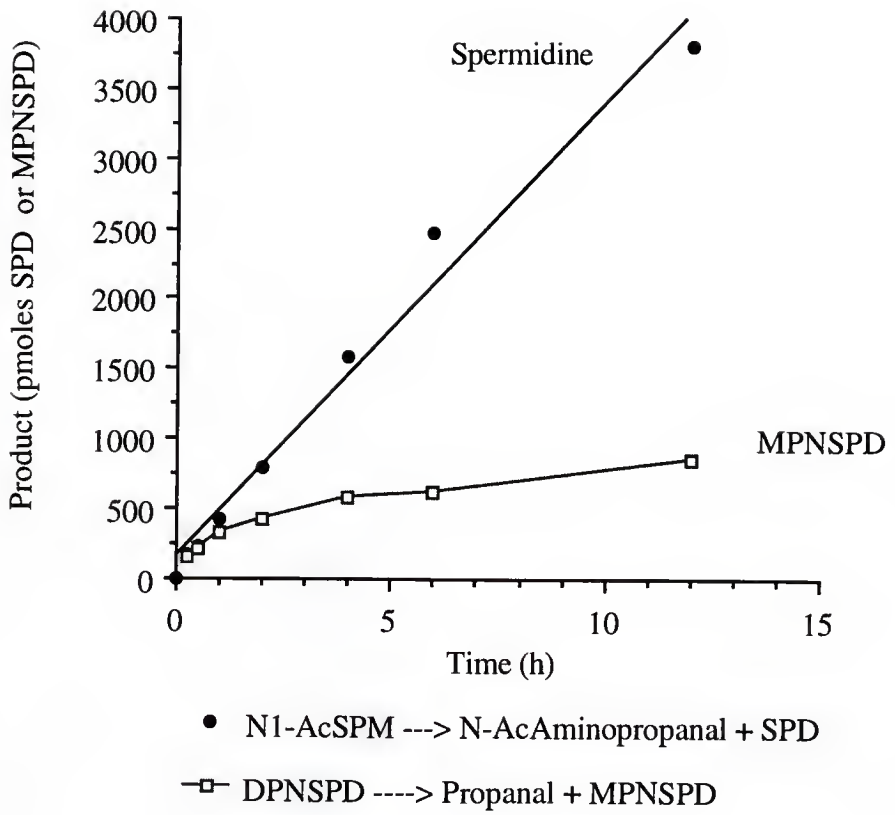


Figure 6-9: Metabolism of N^1-acetylspermine and DPNSPD by partially purified polyamine oxidase in a 12 h period. The reaction product spermidine or MPNSPD was detected by HPLC.

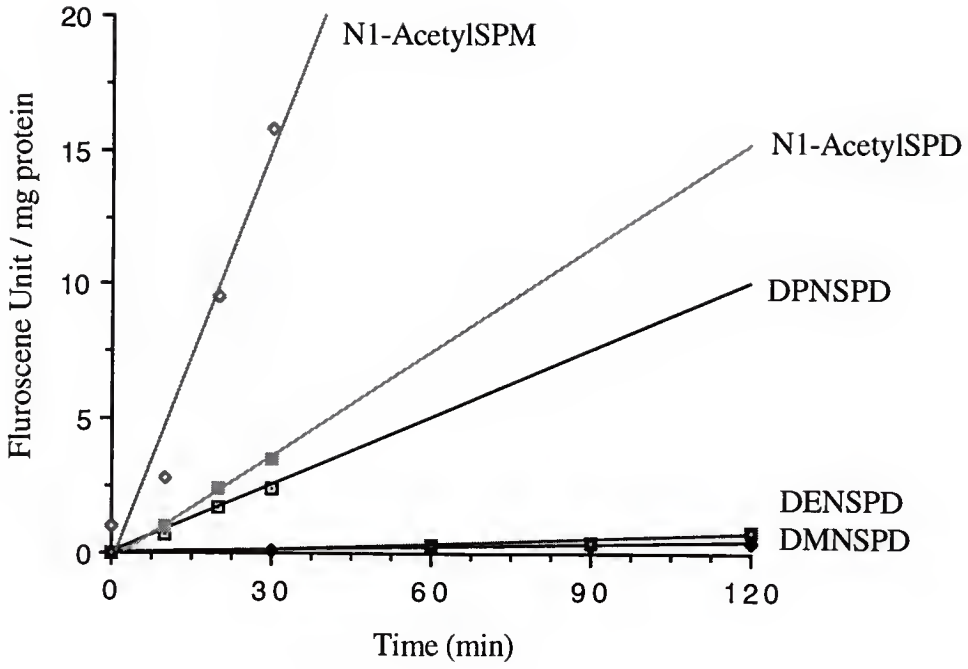


Figure 6-10: Kinetics of the metabolism of different substrates catalyzed by partially purified polyamine oxidase.

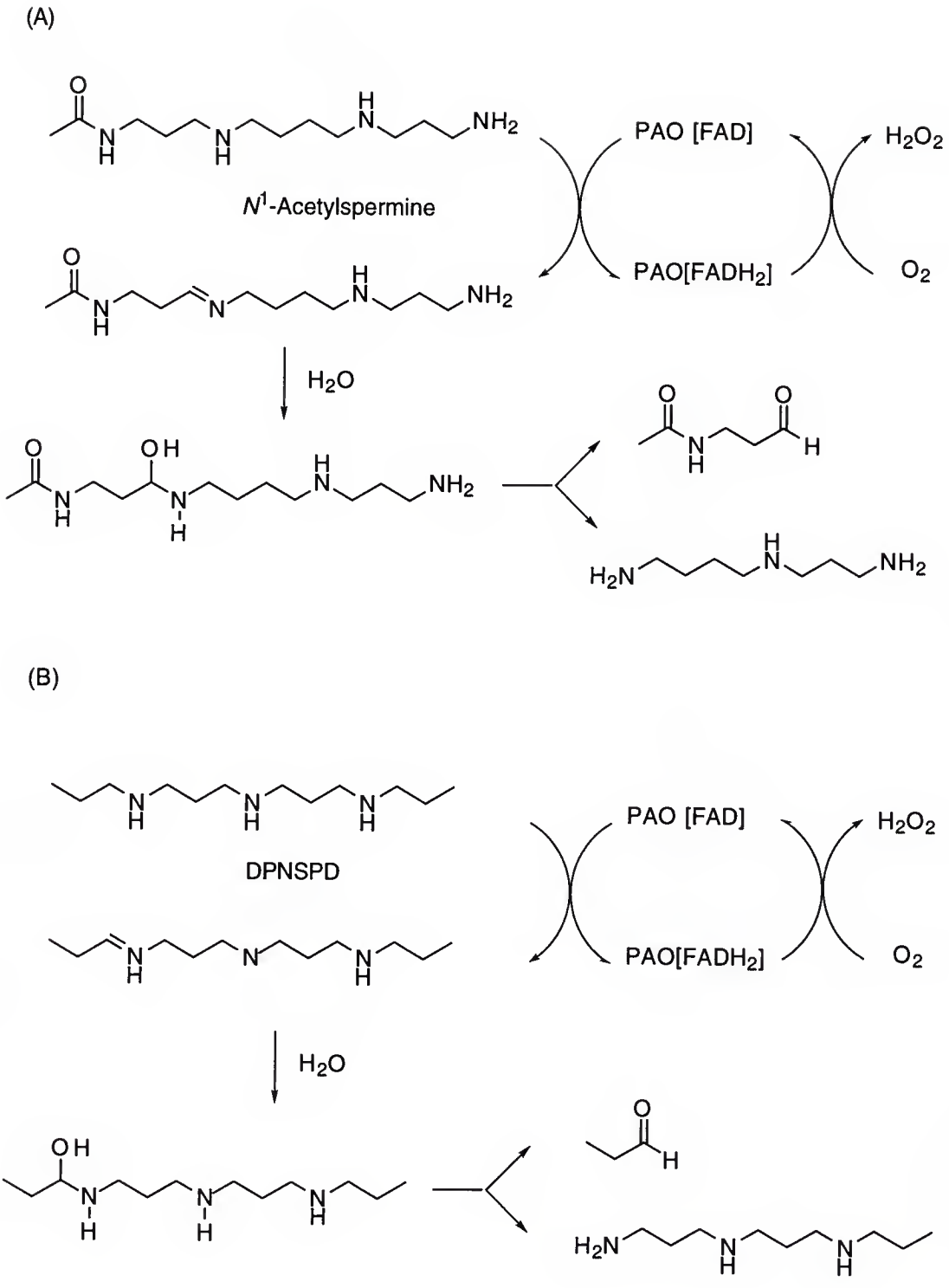


Figure 6-11: Mechanism of polyamine oxidase (PAO) catalyzed reactions.
(A) Deaminopropylation of N^1-acetylspermine by PAO.
(B) Depropylation of DPNSPD by PAO.

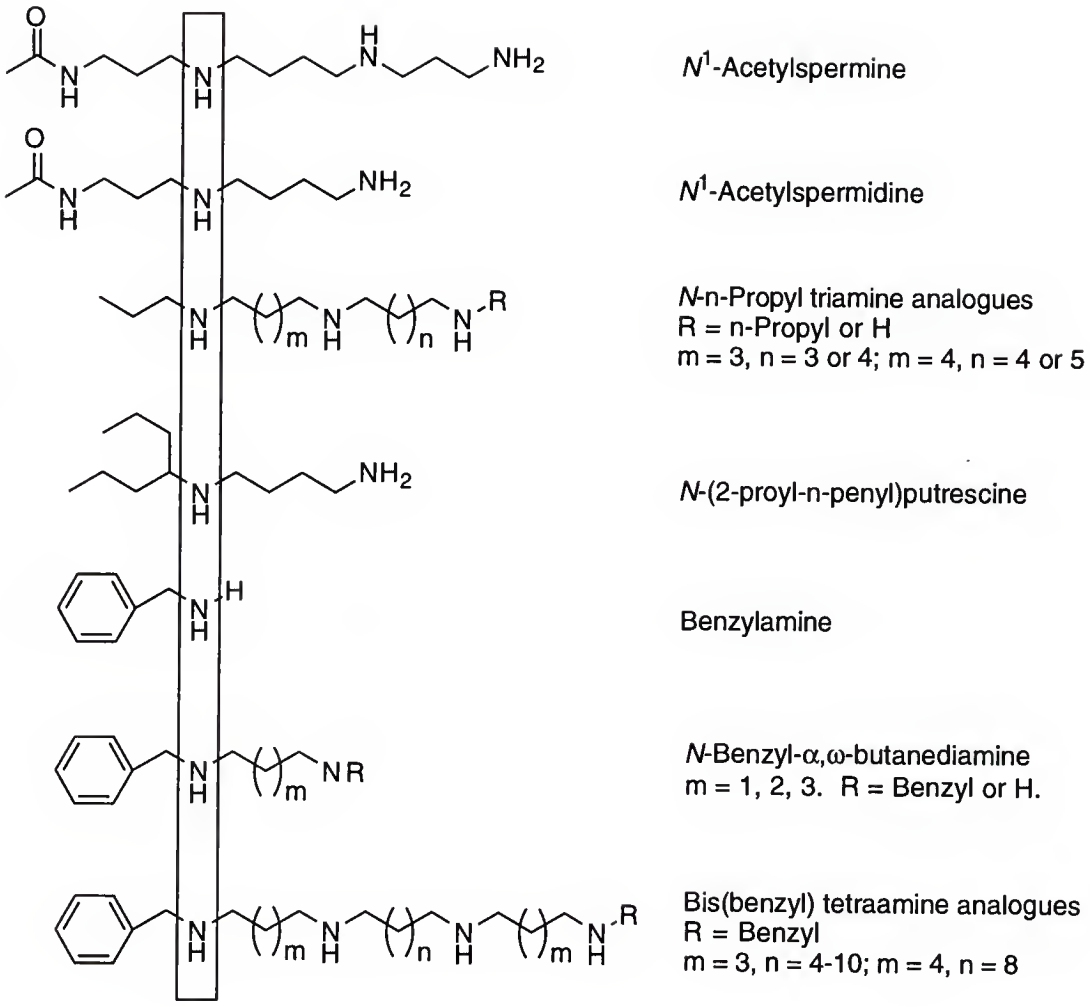


Figure 6-12: Structures of the synthetic polyamine analogues that were demonstrated to be substrates of polyamine oxidase. The structures of N^1-acetylspermine and N^1-acetylspermidine, the natural substrates of PAO, were also included for comparison. The nitrogens attacked by PAO were illustrated in the block.

Table 6-1: Metabolites detected in triamine analogue treated L1210 cells.

Polyamine Analogues	Metabolite	Quantity of Polyamines (nmol/10^6 cells)		
		Parent drug	Metabolite	Metabolite:Parent (%)
Dimethyl triamines				
DMNSPD	nd			
DMSPD	nd			
DMHSPD	nd			
DM(4,5)	nd			
DM(5,5)	nd			
Diethyl Triamines				
DENSPD	MENSPD	3.67	0.147	4%
DESPD	ni			
DEHSPD	ni			
DE(4,5)	nd			
DE(5,5)	nd			
Monopropyl Triamines				
MPNSPD	NSPD	3.07	0.122	4%
MPSPD(N1)	-[a]	3.97	-	-
MPSPD(N8)	-	3.52	-	-
MPHSPD	HSPD	5.16	0.361	7%
Dipropyl Triamines				
DPNSPD	MPNSPD	0.49	0.245	50%
DPSPD	MPSPD(N1)	3.26	0.130	4%
	MPSPD(N8)		0.130	4%
DPHSPD	MPHSPD	3.12	0.374	12%
DP (4,5)	1 metabolite	1.40	0.546	39%
DP (5,5)	nd	0.86		

The polyamine analogues were studied at 100 μM. L1210 cells were incubated for 48 h at 37 °C. All of the metabolites were detected by HPLC. [a]As the metabolite was spermidine, it can not be detected and quantified with the presence of natural spermidine in the cells. nd = non-detectable. ni = non-identified, presented in very little amount (barely above the base line) and hard to identify.

Table 6-2: Metabolism of DPNSPD in different culture systems.

Assay #	Experiment treatments	Metabolites (% of DPNSPD)
1	FBS[a] + L1210 (48 h)	MPNSPD (50%)
2	NuSerum[a] + L1210 (48 h)	MPNSPD (50%)
3	FBS (48 h)	MPNSPD (3%)
4	albumin[b] + L1210 (4 h)	MPNSPD (50%)
5	FBS + bathophenanthroline disulfonic acid (0.1 mM) (48 h)	MPNSPD (0%)
6	FBS + L1210 + bathophenanthroline disulfonic acid (0.1 mM) (48 h)	MPNSPD (50%)

In all of the assays, RPMI-1640 was used as culture media. NuSerum IV is a semi-synthetic FBS substitute, containing 25% of FBS. [a]At concentration of 10%. [b]At concentration of 1.5%.

Table 6-3: Substrate study of rat liver polyamine oxidase.

Substrate	Reaction Product	V_{max} (nmol/min/mg protein)	K_m (μM)
Polyamine analogues with high substrate properties			
N^1-Acetylspermine	Spermidine	2.9	17
N^1-Acetylspermidine	Putrescine	1.4	240
DPNSPD	MPNSPD	1.0	290
Polyamine analogue which is poor substrate			
DENSPD	MENSPD	-[a]	-[a]
Polyamine analogues with no substrate properties			
DMNSPD			
Putrescine			
N^1-Acetylputrescine			
N^8-Acetylspermidine			

V_{max} values were determined by fluorescence assay. The enzyme reactions were studied at pH 7.8. [a]The reaction rate was too low to be accurately measured.

CHAPTER 7
CONCLUSION

Natural occurring polyamines, including spermine, spermidine and putrescine are essential for normal and malignant cell growth. Many studies have shown that polyamine pharmacophore is an excellent candidate for antiproliferative reagents. Previous study of terminal alkylated polyamine analogues have demonstrated that the antiproliferative activity is in the order of: tetraamines > triamines > diamines.

The structure-activity relationship of a series of terminal alkylated tetraamine analogues that were investigated, focused on the role of chain length, terminal nitrogen alkyl group size and the symmetry of the methylene backbone in the antineoplastic properties of polyamine analogues.

First, there are correlations between chain length and IC_{50}'s. For 48 h activity, the norspermines were the least active followed by the spermines, and the homospermines and the homospermine homologues were the most active. This relationship was also observed in 96 h IC_{50}'s [Figure 7-1(a)]. However at 96 h, all of the tetraamine analogues had low IC_{50} values (< 3 μM) and the differences in IC_{50} values between the polyamine analogues were compressed both within and between the respective families.

Second, the number of methylenes separating the amines plays a role in determining polyamine uptake properties. For the methylated, ethylated and propylated tetraamines, the effectiveness with which the analogues compete for uptake follows the trend of spermine spermines ≈ homospermines > DE(3,4,4) ≈ DE(4,5,4) > DE(5,4,5) ≈ norspermine [Figure 7-2 (a)].

Third, a clear correlation is observed between the size of the terminal alkyl substituents and impact on K_i , the parameter reflecting the ability of the analogue to compete with spermidine for the polyamine uptake apparatus. The general trend was that the larger the terminal alkyl group, the larger the K_i value. The steric effects of terminal alkyl groups have substantial impact on K_i values. For example, DIPNSPM and DIPSPM have higher K_i values than DPNSPM and DPSPM, because isopropyl group is sterically more bulky than the linear n-propyl group. In an other case, the ETBHSPM K_i is slightly higher than that of DEHSPM, while the K_i for DTBHSPM is 40 times higher than the DEHSPM. These observations are also keeping with the idea that the polyamine transport apparatus requires only three cationic centers for polyamine recognition and transport.

The structure-activity relationship is further quantitatively investigated by computer-aided molecular modeling. In this model, the K_i value is selected to represent the biological activity. Based on the library of polyamines, including the tetraamine analogues studied above and other series of diamines and triamine analogues, a database containing 72 polyamine analogues was established ($r^2 = 0.65$). This model has been successfully used in design of new polyamine analogues. *A prori* one could not anticipate any quantitative relationship between competition for the polyamine transport apparatus and any of these other biological properties. However, perhaps it not unreasonable to expect that if the molecule designed is recognized by this transport apparatus similarly to analogous with high activity, it would not be surprising to see the candidate has comparable pharmacological properties as the well studied compounds.

In the design of new series of polyamine analogues, dialkylated triamine analogues are selected for their potentially high antineoplastic activity and considerably low toxicity. The triamines were designed and their K_i values were predicted by the computer modeling, suggesting that they should be recognized effectively by the

polyamine transport apparatus. The series of triamine analogues were synthesized and studied for their antiproliferative activities.

The triamine analogues present high antiproliferative activity, especially DE(4,5), very active in both mice leukemia cell line and human melanoma cell line. Although the triamine analogues are less active than the tetraamines during 48 h and 96 h study, the 96 h IC_{50} of dimethyl and diethyl triamines are all in the range of 0.1-10 μM. This kind of high efficacy implies that the triamines can be considered as therapeutic agents.

First, the IC_{50} values are very sensitive to structure modification. At the IC_{50} level, there is an excellent correlation between the length of triamine and the antiproliferative activity (96 h IC_{50}). As shown in Figure 7-1(b), when the terminal groups are fixed, an increase in the length of the methylene backbone from (3,3), (3,4), (4,4) to (4,5), is accompanied by an ascended antiproliferative activity (with the IC_{50} value decreased). The (4,5) triamines are the most active triamine analogues in every case [Figure 7-1(b)]. However, a further elongation of the backbone beyond (4,5) reduces the activity and the (5,5) triamines are less active at both 48 h and 96 h. In addition, the size of the terminal groups also effects the IC_{50} activity [Figure 7-2 (b)]. In general, larger or longer terminal alkyl groups introduces a decrease in the antiproliferative activity, i.e. at 96 h, DMSPD and DESPD are at least twenty times more active than DPSPD. The case for monoalkyl spermidines is very interesting. Alkylation at N^1 results in analogues considerably more active than the alkylation at N^8, although in every case, N^1 and N^8 triamines have very similar K_i values and accumulated to essentially the same intracellular concentrations after treatment. This difference in antineoplastic activity might be related to the fact that N^1-alkylspermidines are elaborated to N^1-alkylspermine conterpartes, tetraamine analogues with higher activity. Recall the 96 h IC_{50} of MESPD(N^1) is 3.0-5.0 μM, while for MESPM the 96 h IC_{50} is 0.33 μM. The N^8-alkylspermidine is not likely to be a substrate of spermine synthase (the enzyme

responsible for spermidine elaboration to spermine) and indeed, no elaboration of MESPD(N^8) is detected in this study.

With the triamines, the relationship between the K_i values and the size of terminal alkyl groups are in agreement with which was observed with the tetraamines. The introduction of terminal alkyl group has a significant impact on the K_i value, i.e. MENSPD has a K_i almost seven times higher than that of the parent amine NSPD, and the K_i of DENSPD is seven times higher than that of MESPD. In general, the order of the ability to compete for the polyamine transport apparatus for different of triamine analogue families falls into the order of (3,4) ≈ (4,4) > (4,5) > (3,3) ≈ (5,5) (Figure 7-2). Compared to the tetraamine analogues, K_i values of triamine analogues are more sensitive to the introduction of alkyl groups to ternimal nitrogens. Again, this is in agreement with the idea that the polyamine transport requires only three cationic centers for polyamine recognition and transport.

The impact of triamine analogues on polyamine pools was also studied. The effects of the triamine analogues on polyamine pools is similar but not the same as the tetraamine analogues. In both cases, putrescine is substantially depleted, accompanied by a moderate decrease in the level of spermidine and with the least effect on spermine concentration. However, when cells are exposed to the same concentration of triamine and tetraamines, triamine analogues are accumulated to a higher concentration than the corresponding tetraamine analogues, although the later deplete polyamine pools to a greater extent, especially in depleting the level of SPM. Tetraamine analogues deplete the overall polyamine pools, while triamine analogues are more selective in depleting spermidine.

The effects of triamine analogues on the polyamine biosynthetic enzymes ODC, AdoMet and on the polyamine catabolizing enzyme SSAT were also investigated. Similar to the tetraamine analogues, the triamines "down regulate" the activity of ODC and AdoMet and "up regulate" the activity of SSAT. For the study of ODC and AdoMet,

the concentration of triamine analogues were at 1 μM, as same as the tetraamine analogues. It is clear that the effects on ODC and AdoMet are less than that of tetraamine analogues. The effect of triamine analogues on SSAT is comparable with that of the tetraamine analogues when a concentration of 10 μM, 5 times of the tetraamine analogues, was selected.

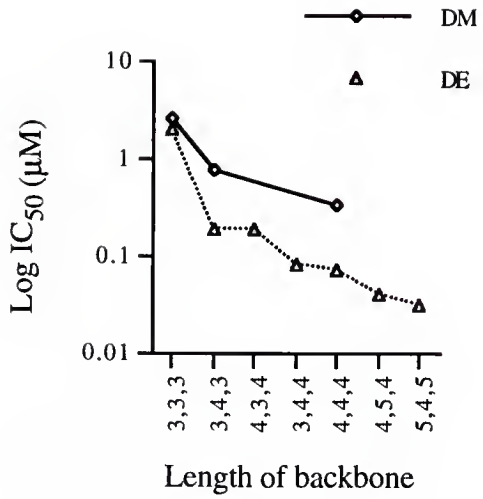
In accordance with our early studies, a conservation of charge with regards to the total polyamine cationic picoequivalence in the triamine treated cells has been observed. For the tetraamine, this phenomenon holds for 24 h but collapses after a period of time and followed by the sign of cytotoxicity. With the triamines, the conservation of charge continues for 48 h, indicating the triamine analogues have less cytotoxicity than the tetraamines.

The animal study on toxicity of triamines further demonstrate that triamine analogous are superior than tetraamine analogues by their low toxicity. The acute toxicities of triamine analogues are only half of those of the corresponding tetraamines. The difference of chronic toxicity between triamines and tetraamines is even more dramatic. Triamines are about 5 to 8 times less toxic than the tetraamines, and this difference is higher than that between the IC_{50}'s. The widening of therapeutic window suggests that triamine analogues can serve as a safer antineoplastic agent .

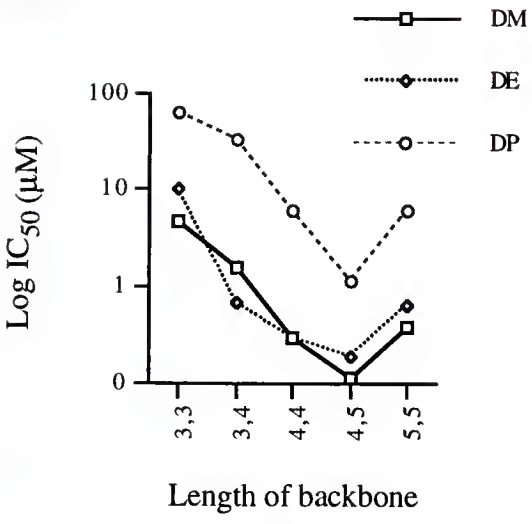
In accordance with several documented N-dealkylation of polyamine analogues in tissues, a new type of N-dealkylation, N-depropylation, was observed for the first time in L1210 cell culture. The N-depropylation happened at a substantial level. After careful studies in cell culture, abundant evidence was gathered showing that the intracellular enzyme(s) was responsible for the N-depropylation. Further investigation of this enzymatic reaction by using partial purified polyamine oxidase (PAO) clearly demonstrated that DPNSPD is a good substrate of rat liver PAO, and DENSPD is a poor substrate, while DMNSPD is not poor substrate or a extremely poor one. The results from these studies both *in vitro* and *in vivo* systems suggest that the PAO may play a very

important role in the observed N-dealkylation of polyamine analogues in animal studies. Understanding of the mechanism of N-dealkylation provides us very important information in drug design.

As the triamine analogues are designed on the base of the information obtained from a library of tetraamines and other polyamine analogous, it is interesting to add these triamines into the previous database and establish a model with almost 100 analogues. Compared to the previous model, the new model (crossvalidated $r^2 = 0.81$) is more accurate in predicting the biological activity of candidate agents, and will provide a very powerful tool in the future drug design.



(a)



(b)

Figure 7-1: The structure-activity relationship between the polyamine analogues and IC_{50} values. (a) Tetraamine analogous. (b) Triamine analogues.

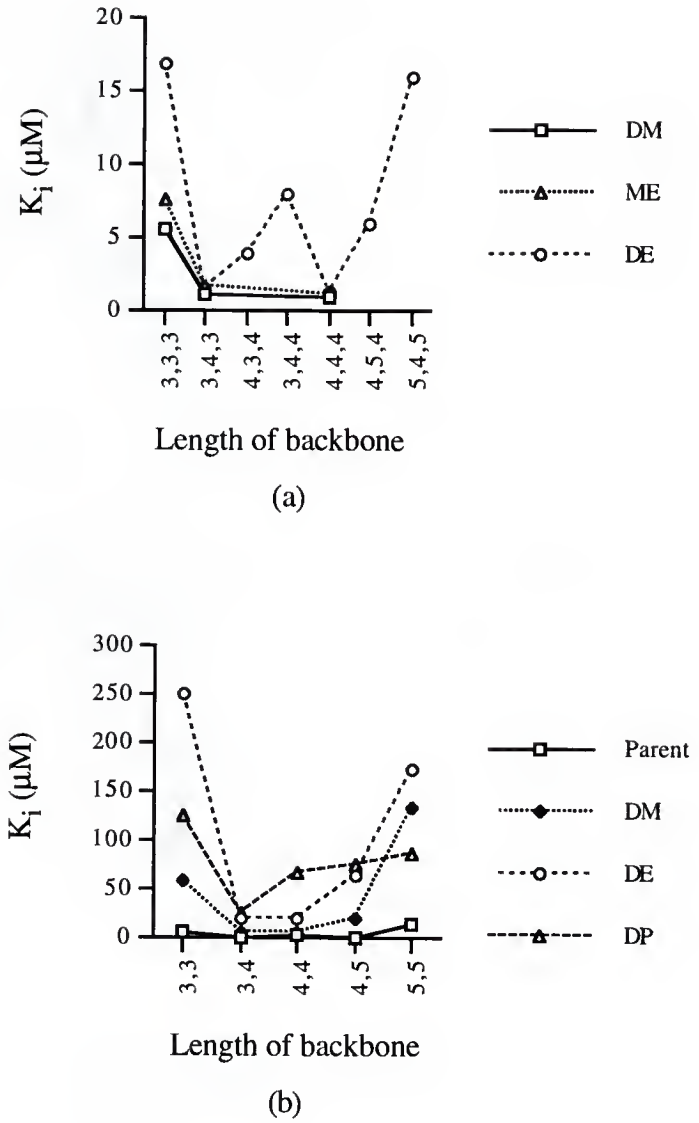


Figure 7-2: The structure-activity relationship between polyamine analogues and K_i values. (a) Tetraamine analogues. (b) Triamine analogues.

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BIOGRAPHICAL SKETCH

Yang Feng was born in Tianjin, the People's Republic of China, on December 27, 1969. She became interested in medicine and chemistry under the influence of her chemist parents. Upon graduation from Tianjin Number One Middle School in Tianjin, in 1988, Yang matriculated at the School of Pharmaceutical Sciences, Beijing Medical University in Beijing, P.R. China, and graduated in 1992 with a Bachelor of Science in medicinal chemistry.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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